(51) International Patent Classification [5] :		(11) International Publication Number:	WO 94/11498
C12N 15/10, C07K 15/14 A61K 37/02, 39/395, G01N 33/68	A1	(43) International Publication Date:	26 May 1994 (26.05.94)

(72) Inventors: and
(75) Inventors/Applicants *(for US only)* : CUMMINGS, Richard, D. [US/US]; 5215 Santa Fe, Edmond, OK 73034 (US). MOORE, Kevin, L. [US/US]; 612 N.W. 42nd Street, Oklahoma City, OK 73118 (US). MCEVER, Rodger, P. [US/US]; 1716 Guilford Lane, Oklahoma City, OK 73120 (US).

(74) Agents: PABST, Patrea, L. et al.; Kilpatrick & Cody, 1100 Peachtree Street, Suite 2800, Atlanta, GA 30309-4530 (US).

(54) Title: GLYCOPROTEIN LIGAND FOR P-SELECTIN AND METHODS OF USE THEREOF

(57) Abstract

P-selectin has been demonstrated to bind primarily to a single glycoprotein ligand on neutrophils and HL-60 cells, when assessed by blotting assays and by affinity chromatography of [^{3}H]glucosamine-labeled HL-60 cell extracts on immobilized P-selectin. This molecule was characterized and distinguished from other well-characterized neutrophil membrane proteins with similar apparent molecular mass. The purified ligand, or fragments thereof, including both the carbohydrate and protein components, or antibodies to the ligand, or fragments or components thereof, can be used as inhibitors of binding of P-selectin to cells.

GLYCOPROTEIN LIGAND FOR P-SELECTIN
AND METHODS OF USE THEREOF

Background of the Invention

The United States government has rights in this
invention as a result of National Institutes of
Health grants HL 34363 (R.P. McEver) and HL 45510
(R.P. McEver and K.L. Moore), CA 38701 (A. Varki),
IT4 RR 05351 (R.D. Cummings), and GM 45914 (D.F.
Smith).

The selectins are three structurally related
membrane glycoproteins that participate in
leukocyte adhesion to vascular endothelium and
platelets, as reviewed by McEver in *Thromb.
Haemostas., 66*: 80-87 (1991) and in *Curr. Opin.
Cell Biol., 4*; 840-849 (1992). P-selectin (CD62),
previously known as GMP-140 or PADGEM protein, is a
receptor for neutrophils, monocytes and subsets of
lymphocytes that is rapidly translocated from
secretory granule membranes to the plasma membrane
of activated platelets, as reported by Hamburger
and McEver, *Blood 75*: 550-554 (1990); Larsen et
al., *Cell 59*: 305-312 (1989) and endothelial cells,
as reported by Geng et al., *Nature, 343*: 757-760
(1990); Lorant et al., *J. Cell Biol., 115*: 223-234
(1991).

E-selectin (ELAM-1) is a cytokine-inducible
endothelial cell receptor for neutrophils, as
reported by Bevilacqua et al., *Proc. Natl. Acad.
Sci. USA, 84*: 9238-9242 (1987), monocytes, as
reported by Hession et al., *Proc. Natl. Acad. Sci.
USA, 87*: 1673-1677 (1990), and memory T cells, as
reported by Picker et al., *Nature (London), 349*:
796-799 (1991); Shimizu et al., *Nature (London),
349*: 799-802 (1991). L-selectin (LAM-1, LECAM-1),
a protein expressed on myeloid cells and most
lymphocytes, participates in neutrophil
extravasation into inflammatory sites and homing of

lymphocytes to peripheral lymph nodes, as reported
by Lasky et al., *Cell, 56*: 1045-1055 (1989);
Siegelman et al., *Science, 243*: 1165-1172 (1989);
Kishimoto et al., *Science, 245*: 1238-1241 (1989);

5 Watson et al., *Nature (London), 349*: 164-167
(1991).

Each selectin functions as a Ca^{2+}-dependent
lectin by recognition of sialylated glycans. Both
E- and P-selectin interact with sialylated,

10 fucosylated lactosaminoglycans on opposing cells,
including the sialyl Le^x tetrasaccharide, as
reported by Phillips et al., *Science, 250*: 1130-
1132 (1990); Walz et al., *Science, 250*: 1132-1135
(1990); Lowe et al., *Cell, 63*: 475-484 (1990);

15 Tiemeyer et al., *Proc. Natl. Acad. Sci. USA, 88*:
1138-1142 (1991); Goelz et al., *Cell, 63*: 1349-1356
(1990); Polley et al., *Proc. Natl. Acad. Sci. USA,
88*: 6224-6228 (1991); Zhou et al., *J. Cell Biol.,
115*: 557-564 (1991). However, the precise

20 carbohydrate structures on myeloid cells recognized
by these two selectins under physiologic conditions
are not known. Such ligands might have unique
structural features that enhance the binding
specificity and/or affinity for their respective

25 receptors.

P-selectin isolated from human platelets binds
with apparent high affinity to a limited number of
sites on neutrophils (Moore et al., *J. Cell Biol.,
112*: 491-499 (1991); Skinner et al., *J. Biol.

30 Chem., 266*: 5371-5374 (1991) and HL-60 cells (Zhou
et al., *J. Cell Biol., 115*: 557-564 (1991)).
Binding is abolished by treatment of the cells with
proteases (Moore et al., (1991)), suggesting that
the glycans on myeloid cells recognized

35 preferentially by P-selectin are on glycoprotein(s)
rather than on glycolipids. The number of binding
sites for platelet P-selectin on neutrophils has

3

been estimated at 10,000-20,000 per cell (Moore et
al., 1991; Skinner et al., 1991), suggesting that
these sites constitute a small component of the
total cell surface protein. The protein portion of
5   this ligand(s) may be crucial for binding by
presenting the glycan in an optimal configuration,
clustering glycans to enhance avidity, favoring the
formation of specific oligosaccharide structures by
cellular glycosyltransferases or modifying enzymes,
10   and/or stabilizing the lectin-carbohydrate
interaction through protein-protein interactions
with P-selectin.
        The potential importance of protein components
in enhancing ligand affinity is supported by
15   studies of CHO cells transfected with a specific
fucosyltransferase (Zhou et al., (1991)). These
cells express higher amounts of the sialyl Le^x
antigen than do HL-60 cells and have protease-
sensitive binding sites for P-selectin. However,
20   the interaction of P-selectin with these sites is
of much lower apparent affinity than with those on
myeloid cells, and adhesion of transfected CHO
cells to immobilized P-selectin is weaker than that
of neutrophils and HL-60 cells (Zhou et al.,
25   (1991)). These observations suggest that myeloid
cells express one or more membrane glycoproteins
not found on CHO cells that enhance the lectin-
mediated interaction with P-selectin.
Alternatively, myeloid cells may express a
30   glycosyltransferase or modifying enzyme not present
in CHO cells.
        It is therefore an object of the present
invention to identify and characterize a specific
glycoprotein ligand for P-selectin (CD62).
35   It is a further object of the present invention
to provide methods and compositions derived from
the characterization of a specific glycoprotein

4

ligand for P-selectin for use in modifying
inflammatory processes and in diagnostic assays.

Summary of the Invention

P-selectin has been demonstrated to bind
primarily to a single glycoprotein ligand on
neutrophils and HL-60 cells, when assessed by
blotting assays and by affinity chromatography of
[³H]glucosamine-labeled HL-60 cell extracts on
immobilized P-selectin. This molecule was
characterized and distinguished from other well-
characterized neutrophil membrane proteins with
similar apparent molecular mass. The amino acid
sequences of some tryptic peptides of the ligand
were determined and found to be unrelated to other
known amino acid sequences.

The purified ligand, or fragments thereof, as
well as carbohydrate and polypeptide components of
the ligand, or antibodies to the ligand or to
fragments thereof, can be used as inhibitors of
binding of P-selectin to cells and in diagnostic
assay 1.

Detailed Description of the Invention

WO 92/01718 entitled "Peptides Selectively
Interacting with Selectins" by Rodger P. McEver,
described the ability of P-selectin (GMP-140) to
mediate cell-cell contact by binding to
carbohydrate ligands on target cells and specific
binding to protease-sensitive sites on human
neutrophils. Studies with antibodies and with
neuraminidase indicated that P-selectin bound to
carbohydrate structures related to sialylated,
fucosylated lactosaminoglycans. As described in WO
92/01718 by Rodger P. McEver, P-selectin was also

5

demonstrated to bind to sialylated, fucosylated
lactosaminoglycans (including the tetrasaccharide
sialyl Lewis x (sLe^x)) on both myeloid and
nonmyeloid cells.

5   The ability of proteases to abolish P-selectin
binding to neutrophils indicated that high affinity
binding of P-selectin to myeloid cells occurred
through interactions with cell surface
glycoprotein(s) rather than with glycolipids. As
10   also described in WO 92/01718 P-selectin bound
preferentially to a glycoprotein in human
neutrophil extracts of Mr 120,000 daltons (D), as
analyzed by SDS-PAGE under reducing conditions.
The glycoprotein was partially purified on a P-
15   selectin affinity column. It appeared to be
heavily glycosylated because it stained poorly with
silver and Coomassie blue. It appeared to be
heavily sialylated because it bound to a wheat germ
agglutinin affinity column. Treatment of the
20   glycoprotein ligand with low doses of sialidase
slowed its mobility on SDS gels, a pattern
consistent with partial desialylation of heavily O-
glycosylated proteins. Binding of P-selectin to
the glycoprotein ligand was Ca^{2+}-dependent, blocked
25   by monoclonal antibodies to P-selectin that also
block P-selectin binding to leukocytes, and
abolished by extensive treatment of the ligand with
sialidase.
        The preferential binding of P-selectin to the
30   120,000 D glycoprotein ligand in myeloid cell
extracts suggested that it contained special
structural features that are recognized with high
affinity by P-selectin. Such structures might not
be present on every protein or lipid characterized
35   by sialylated, fucosylated structures such as sLe^x.
It has now been further demonstrated that the
adhesion of myeloid cells to immobilized P-selectin

6

is much stronger than that to NeoLewis CHO cells (a
cell line expressing sialylated, fucosylated
lactosaminoglycans, described in WO 92/01718), even
though the NeoLewis cells express higher levels of

5   sLe^x antigen, as reported by Zhou et al., *J. Cell
Biol.*, *115*: 557-564 (1991). Furthermore, fluid-
phase [^{125}I]P-selectin binds with high affinity to a
limited number of sites on myeloid cells, whereas
it binds with lower affinity to a higher number of

10   sites on NeoLewis CHO cells. The 120,000 D
glycoprotein ligand for P-selectin in neutrophil
extracts is likely to correspond to the limited
number of protease-sensitive, high affinity binding
sites for P-selectin on intact neutrophils.

15   Interaction of P-selectin with these sites may be
required for efficient adhesion of leukocytes in
flowing blood to P-selectin expressed by activated
platelets or endothelial cells.

A method for purifying the glycoprotein ligand

20   for P-selectin and structural features including
the amino acid sequence of tryptic peptides of the
ligand are described below. The purified ligand,
or fragments thereof, including both the
carbohydrate and protein components, or antibodies

25   to the ligand, or fragments thereof, can be used as
inhibitors of binding of P-selectin to cells.

<u>MATERIALS AND METHODS</u>

<u>Materials</u>

Wheat germ agglutinin (WGA)-agarose, pepstatin,

30   aprotinin, N-acetylglucosamine, leupeptin,
antipain, benzamidine, MOPS, Pipes, BSA, EDTA,
EGTA, and Ponceau S were purchased from Sigma
Chemical Co. (St. Louis, MO).
Diisopropylfluorophosphate, dichloroisocoumarin,

35   Triton X-100 (protein grade), and sialidase
(neuraminidase) from *Arthrobacter ureafaciens* (75
U.mg, EC 3.2.1.18) were obtained from Calbiochem-

7

Behring Corp. (La Jolla, CA), Micro BCA protein
assay kits and Lubrol PX (Surfact Amp PX) were
purchased from Pierce Chemical Company (Rockford,
IL.). Enzymobeads™, Tween-20™, Affigel™-15, and
5   high molecular weight protein standards were from
Bio Rad Laboratories (Hercules, CA). Endo-β-
galactosidase (150 U/mg, EC 3.2.1.103) from
Bacteroides fragilis, 4-methyl-umbelliferyl α-*N*-
acetylneuraminic acid, and 2,3-dehydro-2,3-dideoxy-
10   *N*-acetylneuraminic acid (Neu2en5Ac) were obtained
from Boehringer Mannheim Biochemicals
(Indianapolis, IN). Peptide:*N* glycosidase F
(PNGaseF) from *Flavobacterium meningosepticum* (EC
3.2.2.18, N-glycanase) and endo-α-*N*-
15   acetylgalactosaminidase from *Diplococcus pneumoniae*
(EC 3.2.1.97, O-glycanase™) were purchased from
Genzyme (Cambridge, MA). HBSS was obtained from
Gibco Laboratories (Grand Island, NY). Vecta-Stain
ABC kits were purchased from Vector Laboratories
20   Inc. (Burlingame, CA). Phycoerythrin-streptavidin
was obtained from Becton Dickinson & Co. (San Jose,
CA) and phycoerythrin-conjugated anti-mouse IgG$_1$ was
from Caltag (South San Francisco, CA). Rabbit
anti-mouse IgG was purchased from Organon Teknika
25   (Durham, NC) and protein A-Sepharose CL4B was from
Pharmacia Fine Chemicals (Piscataway, NJ). [6-
^{3}H]glucosamine was obtained from Dupont/New England
Nuclear (Boston, MA). All other chemicals were of
the highest grade available.
30   <u>Antibodies and Proteins</u>
      The anti-P-selectin murine MAbs S12 and G1, and
goat anti-human P-selectin IgG were prepared and
characterized as described by McEver and Martin, *J.
Biol. Chem.*, *259*: 9799-9804 (1984); Geng et al.
35   (1990); Lorant et al. (1991). Rabbit polyclonal
antisera and murine MAbs to human lamp-1 (CD3),
described by Carlsson et al., *J. Biol. Chem.*, *263*:

18911-18919 (1988), and lamp-2 (BB6), Carlsson and
Fukuda, *J. Biol. Chem.*, *264*: 20526-20531 (1989),
and rabbit polyclonal anti-human leukosialin
antiserum, described by Carlsson and Fukuda in *J.*
5   *Biol. Chem.*, *261*: 12779-12786 (1986) were provided
by Dr. Sven Carlsson (University of Umea, Umea,
Sweden). Anti-human leukosialin (CD43) MAb (Leu-
22) was purchased from Becton Dickinson & Co. (San
Jose, CA). The anti-L-selectin murine MAb
10   antibodies DREG-56, DREG-55, and DREG-200,
described by Kishimoto et al., *Proc. Natl. Acad.*
Sci. USA, *87*: 2244-2248 (1990) were provided by Dr.
Takashi Kei Kishimoto (Boehringer Ingelheim
Pharmaceuticals, Inc., Ridgefield, CT). All MAbs
15   are of the IgG_1 subtype and were used in purified
form. Leukosialin purified from HL 60 cells
(Carlsson and Fukuda, 1986) was provided by Dr.
Sven Carlsson (University of Umca). P-selectin was
purified from human platelets as described by Moore
20   et al., (1991). The teachings of these references
are specifically incorporated herein.

Isolation of Human Erythrocyte and Neutrophil
Membranes

Erythrocyte membranes were isolated from
25   leukocyte-depleted human erythrocytes as described
by Rollins and Sims, *J. Immunol.*, *144*: 3478-3483
(1990) and extracted with 0.1 M NaCl, 10 mM MOPS,
pH 7.5, 1% Lubrol™ PX. Detergent-insoluble
material was removed by centrifugation at 16,000 x
30   *g* for 10 min.

Human neutrophils isolated by discontinuous
leukopheresis from volunteer donors were purchased
from the Oklahoma Blood Institute (Oklahoma City,
OK). Each product contained 1.5-3.3 x 10^{10}
35   leukocytes (approximately 85% neutrophils). The
neutrophil product was centrifuged at 200 x *g* for
20 min and the platelet-rich plasma removed.

9

Contaminating erythrocytes were lysed by
resuspending the pellets with 5 mM EDTA, pH 7.5, in
H_2O for 20 s. An equal volume of 1.8% NaCl, 5 mM
EDTA, pH 7.5, was then added to restore

5   isotonicity. The cells were centrifuged at 500 x g
for 5 min and resuspended in ice-cold HBSS
containing 5 mM EDTA and 10 mM MOPS, pH 7.5.
Diisopropylfluorophosphate was then added to a
final concentration of 2 mM and the cell suspension

10   incubated for 10 min on ice. The cells were
centrifuged at 500 g for 5 min at 4°C and
resuspended in ice-cold 100 mM KCl, 3 mM NaCl, 1 mM
Na_2ATP, 3.5 mM $MgCl_2$, 10 mM Pipes, pH 7.3
(relaxation buffer). To this suspension the

15   following protease inhibitors were added at the
indicated final concentrations: 2 mM
diisopropylfluorophosphate, 20 μM leupeptin, 30 μM
antipain, and 1 mM benzamidine. The cell
suspension was pressurized with N_2 at 350 psi in a

20   cell disruption bomb (model 4635; Parr Instrument
Company, Moline, IL.) for 40 min at 4°C with
constant stirring as described by Borregaard et
al., *J. Cell Biol.*, *97*: 52-61 (1983). The cavitate
was collected into EGTA (2 mM final concentration)

25   and nuclei and undisrupted cells were pelleted at
500 g for 10 min at 4°C. The cavitate was
fractionated as described by Eklund and Gabig, *J.
Biol. Chem.*, *265*: 8426-8430 (1990). Briefly, it
was layered over 40% sucrose in relaxation buffer

30   containing 2 mM EGTA, 20 μM leupeptin, 30 μM
antipain, and 1 mM benzamidine, and centrifuged at
104,000 x g (at r_w) for 45 min at 4°C in a rotor
(model SW28; Beckman Instruments, Inc., Palo Alto,
CA). The top layer (FX_1), the 40% sucrose layer

35   (FX_2), and the granule pellet (FX_3) were collected
and assayed for lactate dehydrogenase as a
cytoplasmic marker, alkaline phosphatase as a

plasma membrane marker, and myeloperoxidase as a
marker for azurophilic granules as described by
Borregaard et al., (1983); Geng et al., (1990).

Table I shows the distribution of marker enzymes
5   in the various fractions. FX_2, enriched for
alkaline phosphatase, was diluted with four volumes
of 0.1 M NaCl, 10 mM MOPS, pH 7.5, and centrifuged
at 111,000 x g (at r_{av}) for 60 min at 4°C in a rotor
(model 50.2 Ti; Beckman Instruments, Inc.). The
10   supernatant was collected and the membrane pellet
was extracted with 1% Lubrol™ PX, 0.1 M NaCl, 10 mM
MOPS, pH 7.5, 0.02% sodium azide, 20 μM leupeptin,
30 μM antipain, 1 mM benzamidine, and stored at
4°C.

15   HL-60 cells, maintained in suspension culture in
RPMI-1640 supplemented with 10% FCS, 100 IU/ml
penicillin, and 100 μg/ml streptomycin, were washed
in HBSS, 10 mM MOPS, pH 7.5, and membranes were
isolated exactly as described for neutrophils.

20   <u>Partial Purification of P-selectin Ligand</u>
Neutrophil or HL-60 cell membrane extracts were
applied to a wheat germ agglutinin (WGA) affinity
column (0.9 x 20 cm. 7.6 mg lectin/ml resin)
equilibrated at room temperature with 0.5 M NaCl,
25   10 mM MOPS, pH 7.5, 0.02% sodium azide, 0.1%
Lubrol™ PX. The column was washed with five column
volumes of equilibration buffer, followed by two
column volumes of 0.1 M NaCl, 10 mM MOPS, pH 7.5, 5
mM EDTA, 0.02% sodium azide, 0.01% Lubrol™ PX. The
30   column was then eluted with the above buffer
containing 100 mM N-acetylglucosamine. Protein-
containing fractions were pooled and extensively
dialyzed against 0.1 M NaCl, 10 mM MCPS, pH 7.5,
0.02% sodium azide, 0.01% Lubrol™ PX at 4°C. The
35   dialyzed WGA column eluate was made 1 mM in $CaCl_2$
and $MgCl_2$ and applied to a human serum albumin
Affigel™ 15 precolumn (0.9 x 11 cm, 25 mg

protein/ml resin) hooked in series to a P selectin-
Affigel 15™ column (0.6 x 13 cm, 2 mg protein/ml
resin). The columns were equilibrated with 0.1 M
NaCl, 10 mM MOPS, pH 7.5, 1 mM CaCl$_2$, 1 mM MgCl$_2$,

5   0.02% sodium azide, 0.01% Lubrol™ PX. After the
samples were applied the columns were washed with
100 column volumes of equilibration buffer, and
eluted with equilibration buffer containing 5 mM
EDTA. Yields were estimated by protein assays with

10   the Micro BCA protein assay kit using BSA as a
standard.

12

Table I. Distribution of Marker Enzymes from
Subcellular Fractions of Nitrogen-cavitated Human
Neutrophils.

	Lactate dehydrogenase	Myeloperoxidase	Alkaline phosphatase
FX$_1$ (cytosol)	95.6 ± 0.5	0	29.0 ± 2.7
FX$_2$ (membrane)	4.1 ± 0.5	2.6 ± 1.0	56.8 ± 8.7
FX$_3$ (granule)	0	97.4 ± 1.0	14.1 ± 5.5

Results are expressed as the percentage of the
total enzyme activity in the cavitate (mean ± SD, n
= 3).

P-selectin Blotting Assay

Samples were electrophoresed on 7.5% SDS
polyacrylamide gels (SDS-PAGE) and proteins
electrophoretically transferred to Immobilon-P™
membranes (Millipore Corp., Bedford, MA) for 4-5 h
at 0.5 A. The positions of the molecular weight
standards were marked with a pen after staining the
membranes with Ponceau S. The membranes were
blocked overnight at 4°C in 0.1 M NaCl, 10 mM MOPS,
pH 7.5, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.02% sodium azide,
10% (wt/vol) Carnation™ nonfat dry milk, and then
washed with the same buffer containing 0.1% Tween-
20 without milk. The membranes were incubated with
[^{125}I]P-selectin (0.5-1.0 nM), iodinated as described
by Moore et al., 1991, using standard techniques,
in 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM CaCl$_2$, 1 mM
MgCl$_2$, 0.05% Lubrol™ PX, 1% human serum albumin for
1 h at room temperature. After extensive washing
the membrane was dried and exposed to Kodak X-OMAT
AR™ film (Eastman Kodak Ccmpany, Rochester, NY) for
6 18 at -70°C.

Metabolic Radiolabeling of HL-60 Cells and
Isolation of [³H]glucosamine-labeled P-selectin
Ligand

    HL-60 cells (1-2 x 10⁶ cells/ml) in 100-mm tissue
5   culture dishes were labeled for 48 h with 50 µCi/ml
[6-³H]glucosamine at 37°C in RPMI-1640 containing
10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and
100 µg/ml streptomycin. At the end of the labeling
periods the cells were washed three times by
10   centrifugation and resuspension in ice-cold PBS.
The cell pellet was solubilized with 0.1 M NaCl, 10
mM MOPS, pH 7.5, 4 mM CaCl₂, 4 mM MgCl₂, 1% Triton
X-100™, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 8
µg/ml pepstatin, 2 mM PMSF, 10 mM benzamidine, and
15   0.5 mM dichloroisocoumarin. The solubilized cells
were allowed to sit on ice for 1-2 h and then
sonicated for 20 min at 4°C in a water bath
sonicator. The cell extract was centrifuged for 5
min at 16,000 x g and the supernatant was applied
20   to a P-selectin-Affigel 15™ column (0.25 x 13 cm, 2
mg protein/ml resin) equilibrated with 0.1 M NaCl,
10 mM MOPS, pH 7.5, 2 mM CaCl₂, 2 mM MgCl₂, 0.1%
Triton X-100. The column was washed with 10-20
column volumes of equilibration buffer and bound
25   material was eluted with equilibration buffer
containing 10 mM EDTA. Fractions (1 ml) were
collected and monitored for radioactivity by liquid
scintillation counting. Samples of the run-through
fractions and the bound, EDTA-eluted fractions were
30   analyzed by SDS-PAGE under reducing conditions and
fluorography. A single major glycoprotein of
approximately 120 kD was isolated. In non-reducing
SDS-PAGE, this glycoprotein species had a mobility
corresponding to approximately 250 kD. Treatment
35   of the 120 kD glycoprotein with neuraminidase
abolished binding to P-selectin and caused an
unusual decrease in electrophoretic mobility of the

protein in SDS-PAGE. This result demonstrates that
the HL-60 ligand can be purified free of any
contaminating glycoproteins. By this technique,
approximately 50,000 cpm of radiolabeled ligand can
5   be obtained from a single culture dish of cells.

Analysis of [³H]glucosamine-labeled P-selectin
Ligand

Metabolically labeled proteins eluted from the
P-selectin column (above) were precipitated in the
10   presence of 0.1 mg/ml BSA by addition of cold TCA
(10% final concentration). The resulting pellets
were washed with 1 ml acidified acetone (0.2%),
solubilized in 0.1 M NaOH and electrophoresed under
reducing and nonreducing conditions on 10% SDS-
15   polyacrylamide gels. The gels were stained with
Coomassie blue and then processed for fluorography
with EN³HANCE™ (Dupont/New England Nuclear, Boston,
MA) according to the manufacturer's instructions.
The dried gels were then exposed to Kodak X-OMAT
20   AR™ film at -80°C.

Enzyme Digestion

In certain experiments, samples analyzed by P-
selectin blotting were pretreated with exo- or
endo-glycosidases before SDS-PAGE. For sialidase
25   and endo-β-galactosidase digestions of P-selectin
ligand, samples were dialyzed against 0.15 M NaCl,
50 mM acetate, pH 6.0, 9 mM $CaCl_2$, 0.02% azide,
0.01% Lubrol™ PX, and incubated for various times
at 37°C in the presence or absence of 200 mU/ml of
30   enzyme. For PNGaseF and endo-α-N-
acetylgalactosaminidase digestions, samples were
first reduced and denatured by boiling in 0.5% SDS,
0.5% β-mercaptoethanol for 5 min, and then a 7.5-
fold molar excess of NP-40 was added. The samples
35   were incubated for 16 h at 37°C with either PNGaseF
(20 U/ml at pH 8.6) or endo-α-N

15

acetylgalactosaminidase (70 mU/ml at pH 6.5) in the
presence of 5 mM PMSF and 5 mM 1,10 phenanthroline.

Affinity purified [^{3}H]glucosamine-labeled P-
selectin ligand was incubated for 24 h in 25 mM
5   sodium acetate, pH 5.5 at 37°C under a toluene
atmosphere in the presence or absence of 1 U/ml of
A. ureafaciens sialidase for 18 h. For PNGaseF
digestion of metabolically labeled ligand, samples
were denatured by boiling in 0.25% SDS, 25 mM β-
10   mercaptoethanol for 5 min, and NP-40 was added in
eight-fold excess (wt/wt) over SDS. The samples
were incubated for 24 h with PNGaseF (3.3 U/ml) in
a toluene atmosphere. The samples were then
precipitated with TCA and subjected to SDS-PAGE and
15   fluorography as described above.

Flow Cytometry

Human neutrophils, isolated as described by
Hamburger and McEver, (1990), were suspended
(10^6/ml) in HBSS containing 1% FCS and 0.1% sodium
20   azide (HBSS/FCS/Az). 1 ml of neutrophil suspension
was underlaid with 100 μl FCS and centrifuged at
500 g for 5 min. The neutrophil pellet was
resuspended in 50 μl of purified P selectin (10
μl/ml, in HBSS/FCS/Az), and then incubated
25   sequentially with 50 μl of biotin-conjugated S12
(10 μg/ml, in HBSS/FCS/Az) and 20 μl of
phycoerythrin-streptavidin (neat). In certain
experiments, the neutrophils were preincubated for
10-15 min with antisera or antibodies before the
30   addition of P-selectin. Between each step the
cells were diluted with one ml of HBSS/FCS/Az,
underlaid with 100 μl FCS, and centrifuged at 500 g
for 5 min. All steps were performed at 4°C. After
the last wash, the cells were fixed with 1 ml of 1%
35   paraformaldehyde in HBSS and analyzed in a FACScan™
flow cytometer Becton Dickinson & Co., Mountain
View, CA) formatted for two color analysis as

16

described by Moore, et al., (1991). Binding of P-
selectin to intact neutrophils as assessed by this
assay was Ca^{2+}-dependent, was blocked by G1, and
was abolished by pretreatment of the cells with

5   trypsin or sialidase.

Immunoprecipitations

WGA eluate was incubated with 10 μg of anti-
leukosialin (Leu22) or an isotype matched control
monoclonal antibody for 1 h at 37°C. The mixture

10   was then incubated with protein A-Sepharose™ CL4B
beads saturated with rabbit anti-mouse IgG for 1 h
at 37°C. The beads were pelleted, washed four
times with 1 ml of 0.1 M NaCl, 20 mM Tris, pH 7.5,
1% Triton X-100™, and bound material eluted by

15   boiling 5 min in 2% SDS, 60 mM Tris, pH 6.8, and 5%
ß-mercaptoethanol. Immunoprecipitates and
immunosupernatants were then analyzed by P-selectin
blotting and by Western blotting using Leu22 as a
probe.

20   Assay of Sialidase Activity in Commercial Enzyme
     Preparations

The sialidase activity in O-glycanase (endo-α N-
acetylgalactosaminidase) or *A. ureafaciens*
sialidase was assayed by incubation of dilutions of

25   the enzymes with 50 nmol 4-methyl-umbelliferyl-α-N-
acetylneuraminic acid in 50 μl of sodium
cacodylate, pH 6.5, 10 mM calcium acetate, for
various time periods. Incubations were quenched by
addition of 0.95 ml 0.1 M sodium bicarbonate, pH

30   9.3, and assayed for released 4-methylumbelliferone
by fluorescence (excitation = 365 nM, emission =
450 nM).

     Purification of P-selectin Ligand for Sequencing
     and Preparation of a Radioiodinated Form

35   Neutrophil membranes were extracted with 5%
Triton X-100™, 0.1 M NaCl, 10 mM MOPS, pH 7.5,
0.02% sodium azide, 20 μM leupeptin, 30 μM

17

antipain, 1 mM benzamidine, and the extract applied
to a wheat germ agglutinin (WGA) affinity column
(1.5 x 20 cm, 7.6 mg lectin/ml resin, Vector
Laboratories), equilibrated at room temperature

5   with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA,
0.02% sodium azide, 1% Triton X-100™. The column
was washed with two column volumes of equilibration
buffer, followed by four column volumes of 0.1 M
NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% sodium

10   azide, 0.2% Brij-58. The column was then eluted
with the above buffer containing 500 mM N-
acetylglucosamine. Protein-containing fractions
were pooled and subjected to an additional affinity
chromatographic step using a P-selectin-Emphaze™

15   column. The pooled fractions were made 8 mM in
CaCl$_2$ and applied to the P-selectin-Emphaze™ column
(0.6 x 14 cm, 7.5 mg protein/ml resin) equilibrated
with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM CaCl$_2$, 2
mM MgCl$_2$, 0.02% sodium azide, 0.02% Brij-58.

20   Recombinant truncated P-selectin (tPS, see Ushiyama
et al., J. Biol. Chem., 268: 15229-15237 (1993))
was coupled to 3M Emphaze™ Biosupport Media (Pierce
Chemical Co.) according to the manufacturer's
instruction in 0.1 M MOPS, pH 7.5, 0.8 M Na

25   citrate. The column was washed with 10 column
volumes of equilibration buffer, and eluted with
equilibration buffer containing 5 mM EDTA. The tPS
column was loaded at a flow rate of 0.1 ml/min,
washed at 1 ml/min and eluted at 0.1 ml/min.

30   P-selectin ligand-containing fractions eluted
from the tPS-Emphaze™ column were pooled and loaded
onto an anion exchange column, Mono Q PC 1.6/5
column equilibrated with 0.1 M NaCl, 20 mM MOPS, pH
7.5, 2 mM EDTA, 0.02% sodium azide, 0.02% Brij-58

35   using a SMART™ Micro Separation System
(Pharmacia/LKB). The sample was loaded at 0.1
ml/min, washed with several column volumes of

equilibration buffer, and then developed with a 2
ml linear gradient of NaCl (0.1 M - 1.0 M NaCl) at
50 μl/min. Ligand-containing fractions were
assessed for purity by SDS-PAGE and autoradiography
5   after iodination of aliquots of the fractions using
IodoBeads™ (Pierce) according to the instructions
of the manufacturer. Under non-reducing conditions
a major band of 250 kD was observed, whereas under
reducing conditions a major band at 120 kD was
10 visible. These results demonstrate that the ligand
for P-selectin was purified from human neutrophils.

For studies using iodinated P-selectin ligand,
the ^{125}I-labeled ligand was subjected to gel
filtration on a Superose™ 6 column. The
15 availability of highly purified [^{125}I]P-selectin
ligand allowed various functional and structural
analyses to be carried out on the ligand.
Typically, 75 to 90 percent of the [^{125}I]P-selectin
ligand re-bound to a P-selectin-immobilized
20 affinity column (see above) and was eluted with
EDTA. This material specifically bound to
recombinant soluble P-selectin immobilized on
microtiter plates in both a time- and dose-
dependent fashion. Binding was abolished with EDTA
25 and anti-P-selectin monoclonal antibodies which
inhibit P-selectin function, but not anti-P-
selectin monoclonal antibodies which do not inhibit
function. These data show that the function of the
ligand was not substantially altered by
30 radioiodination.

        P-selectin Ligand Amino Acid Sequencing
        Ligand-containing fractions from the Mono Q PC
1.6/5 column (above) were pooled, then diluted with
2 parts HPLC grade H$_2$O and centrifuged for 10 min at
35 16,000 x g. The sample was applied to a Prospin™
Sample Preparation Cartridge (Applied Biosystems)
after wetting the PVDF membrane with HPLC grade

19

methanol. The cartridge was centrifuged at 4500 x
g for one hour in a Fisher Model 59A Microfuge
equipped with a swing-out rotor. After the sample
was applied, the PVDF membrane was washed twice
5   with 400 μl of HPLC grade H_2O. The PVDF membrane
was removed using a Prospin™ Membrane Removal Punch
(Applied Biosystems) and washed ten times with 1 ml
of HPLC grade H_2O. After the last wash was removed,
the PVDF membrane was frozen on dry ice.
10   The sample (designated gp120) was shipped on dry
ice to Harvard Microchem (16 Divinity Avenue,
Cambridge, MA 02138) for N-terminal sequencing and
in situ trypsin digestion and HPLC separation of
peptides.
15   RESULTS
     Identification of a P-selectin Ligand
          To identify proteins from myeloid cells which
bind P-selectin, neutrophil and HL-60 cell membrane
extracts were electrophoresed on 7.5% SDS-
20   polyacrylamide gels, transferred to Immobilon™
membranes, and probed with [^{125}I]P-selectin. When
samples were analyzed without reduction, P-selectin
bound preferentially to a glycoprotein species with
an approximately 250,000 M_r from both neutrophil and
25   HL-60 cell membranes as determined by SDS-PAGE.
Cell membrane extracts (80 μg protein/lane) were
electrophoresed on 7.5% SDS-polyacrylamide gels
under nonreducing or reducing conditions,
transferred to Immobilon™ membranes, and probed
30   with [^{125}I]P-selectin. Under nonreducing conditions
P-selectin also bound to proteins at the stacking
gel interface and to a minor species with an
approximately 160,000 M_r. When samples were
analyzed after reduction, P-selectin preferentially
35   bound to a glycoprotein with an approximately
120,000 M_r. Minor bands were observed at
approximately 250,000 and approximately 90,000 M_r.

20

Under both reducing and nonreducing conditions P-
selectin also bound to the blots at the dye front.
P-selectin binding proteins were not detected when
an equivalent amount of erythrocyte membrane
5   protein was analyzed in parallel. The total
proteins in the neutrophil cavitate were also
solubilized with SDS and analyzed for their ability
to interact with P-selectin with the blotting
assay. P-selectin bound only to proteins with
10   apparent molecular weights of 120,000 and 90,000
under reducing conditions. Although the
sensitivity of this analysis was limited by the
amount of protein that could be run on the gel, the
results indicate that major ligands that were
15   either not enriched in the membrane fraction (FX$_2$)
or not effectively solubilized by nonionic
detergent were not excluded.

         To further assess the specificity of the
blotting assay, neutrophil membrane extracts
20   electrophoresed under reducing conditions were
probed with [^{125}I]P-selectin in the presence or
absence of EDTA or anti-P-selectin MAbs.
Neutrophil membrane extracts (200 μg protein/lane)
were electrophoresed on 7.5% SDS-polyacrylamide
25   gels under reducing conditions, transferred to
Immobilon membranes, and probed with [^{125}I]P-selectin
alone, in the presence of 10 mM EDTA, or in the
presence of 20 μg/ml of the anti-P selectin MAbs G1
or S12. [^{125}I]P-selectin binding to the major 120-kD
30   and the minor 250-kD species was Ca^{2+}-dependent, a
characteristic of all selectin-dependent cellular
interactions. Binding to both species was also
blocked by G1, a MAb to P-selectin that inhibits
adhesion of myeloid cells to P-selectin, but not by
35   S12, a MAb to P-selectin that does not block
adhesion. Binding of [^{125}I]P-selectin was also
inhibited by a 100-fold excess of unlabeled P-

selectin. The binding of [^{125}I]P-selectin to the dye
front and to the 90,000 D protein was not blocked
by EDTA or G1, suggesting that these interactions
were nonspecific or used a specific Ca^{2+}-independent
5   recognition mechanism.

Purification of P-selectin Ligand from
Neutrophils

Neutrophils were disrupted and the membrane
fraction (FX$_2$) isolated by fractionation of the
10   cavitate as described in Materials and Methods.
The membrane fraction constituted approximately 5%
to 7% (n>10) of the protein in the cavitate. This
fractionation depleted both cytosolic proteins and
azurophilic granules as shown by Table I. Proteins
15   binding P-selectin were not detected in the
cytosolic fraction (FX$_1$) with the blotting assay.
The final membrane pellet was solubilized with
nonionic detergent and applied to a WGA column
which bound 4-5% of the protein in the membrane
20   extract. P-selectin blotting assays of reduced
proteins demonstrated that both the major 120,000 D
and the minor 250,000 D ligands bound
quantitatively to WGA. However, the 90,000 D band
and the band at the dye front observed in the
25   membrane extract were not bound by WGA. After
extensive dialysis, the WGA eluate was applied to
an Affigel 15™ precolumn in series with a P-
selectin affinity column. Approximately 2% of the
protein in the WGA eluate bound to the P-selectin
30   column and could be eluted with EDTA. Both the
250,000 D and the 120,000 D ligands bound
quantitatively to the P-selectin column.
Quantitative analysis of the protein recovered from
the P-selectin eluate indicated that the ligand(s)
35   formed less than 0.01% of the total protein in the
neutrophil cavitate. Elution of bound proteins
from the P-selectin column with EDTA demonstrated

22

that the interaction of nondenatured neutrophil
ligands with P-selectin was also Ca^{2+}-dependent.
Neither species was eluted from the Affigel™ 15
precolumn with EDTA.

5   A silver-stained SDS-polyacrylamide gel of
proteins from the various stages in the partial
purification procedure was run under reducing
conditions. Samples from the indicated steps of
the isolation procedure were electrophoresed on
10   7.5% SDS-polyacrylamide gels under reducing
conditions, transferred to Immobilon™ membranes,
and probed with [^{125}I]P-selectin. The amounts of
protein loaded onto the lanes were as follows:
membrane extract and WGA flow through, 200 μg; WGA
15   eluate and P-selectin flow through, 50 μg; P-
selectin eluate, 2 μg. The same samples (10 μg
protein/lane) were also analyzed by SDS-PAGE under
the reducing conditions followed by silver
staining. The major silver-stained band in the P-
20   selectin eluate had an approximately 150,000 M_r
which is similar to that of P-selectin itself. To
determine whether this protein represented P-
selectin that had leached off the P-selectin
column, the P-selectin eluate was analyzed by SDS-
25   PAGE under both reducing and nonreducing
conditions, followed by silver staining, Western
blotting with goat anti-P-selectin IgG, and P-
selectin blotting. The major silver-stained
protein in the P-selectin eluate was indeed P-
30   selectin. Purified P-selectin migrates with an
approximately 120,000 M_r under nonreducing
conditions; a minor component migrates with an
approximately 250,000 M_r. After reduction the
protein migrates more slowly with an approximately
35   150,000 M_r. The two nonreduced bands and the one
reduced band detected by silver staining of the P-
selectin eluate co-migrated with purified P-

selectin and were recognized by anti-P-selectin
IgG. The P-selectin ligand identified in the
blotting assay was not detected by silver staining
and migrated differently than P-selectin under both
5   reducing and nonreducing conditions. When the P-
selectin eluate was electrophoresed without
reduction, P-selectin did not bind to proteins at
the stacking gel interface. Therefore, the P-
selectin binding proteins at the stacking gel
10   interface, observed in extracts of neutrophil
membranes, were probably an artifact due to the
relatively high amount of protein loaded on the
gel.

Characterization of the P-selectin Ligand from
15   Neutrophils

The ligand on intact target cells requires
sialic acids to interact with P-selectin. To
determine whether the ligand detected by blotting
of neutrophil membranes contained sialic acids that
20   were essential for recognition by P-selectin,
neutrophil membrane glycoproteins which bound to
WGA were treated with sialidase (200 mU/ml) for
varying times before SDS-PAGE under reducing
conditions and then analyzed for their ability to
25   bind P-selectin. Neutrophil WGA eluate (50 μg) was
either sham-treated or digested with 200 mU/ml of
sialidase or with 20 U/ml of PNGaseF for 16 h, then
electrophoresed on 7.5% SDS polyacrylamide gels
under reducing conditions, transferred to Immobilon
30   membranes, and probed with [^{125}I]P-selectin.
Sialidase digestion for 30 min increased the
apparent molecular weight of the major 120,000 D
ligand, a shift characteristic of heavily
sialylated glycoproteins. Longer sialidase
35   digestion did not further alter the electrophoretic
mobility of the ligand but did abolish its ability

24

to bind [^{125}I]P-selectin. Sialidase treatment had a
similar effect on the minor 250 kD ligand.
            These results demonstrate that the ligand
contains sialic acid residues that are critical for
5   recognition by P-selectin, but suggest that only a
portion of the sialic acid residues are required
for binding.

            To examine whether the ligand contained N-linked
glycans, neutrophil membrane glycoproteins which
10   bound to WGA were digested with PNGaseF. This
treatment did not affect [^{125}I]P-selectin binding but
did decrease the apparent molecular weight of the
ligand by approximately 3000 D, consistent with the
enzymatic removal of one or two N-linked glycan
15   chains. This demonstrates that the ligand contains
at least one N-linked oligosaccharide chain that is
not required for P-selectin binding. Although one
could not directly assess whether N-linked glycans
were quantitatively removed from the ligand,
20   conditions that normally cleave such glycans from
most proteins were used.

            Prolonged treatment of neutrophil membrane
extracts with endo-α-N-acetylgalactosaminidase (O-
glycanase) abolished binding of [^{125}I]P-selectin in
25   the blotting assay, whereas sham digestion was
without effect. This was not expected, since only
nonsialylated Galβ1-3GalNAc disaccharides O-linked
to serine or threonine residues are known
substrates for the enzyme. Assays using a
30   synthetic sialidase substrate confirmed the
presence of a small amount of sialidase (0.01 mU/mU
O-glycanase) contaminating the O-glycanase.
Although the level of activity was small, it was
stable to prolonged incubations under the
35   conditions recommended by the manufacturer for use
of the O-glycanase preparation. To prove that the
contaminating sialidase was responsible for the

loss of P-selectin binding, the digestions were
repeated in the presence of a competitive sialidase
inhibitor, Neu2en5Ac. Under these conditions endo-
α-N-acetylgalactosaminidase digestion had no effect
5   on [^{125}I]P-selectin binding to the ligand or the
apparent molecular weight of the ligand. Because
the ligand requires sialic acid to interact with P-
selectin, the blotting assay could not be used to
assess the role of O-linked glycans in recognition
10   by P-selectin.

Isolation of a P-selectin Ligand from
Metabolically Labeled HL-60 Cells

P-selectin blotting of denatured membrane
proteins from myeloid cells may not detect
15   molecules whose ability to bind P-selectin is
dependent on secondary and/or tertiary structure.
As an independent approach to identify ligands for
P-selectin, HL-60 cells were metabolically labeled
with [^{3}H]glucosamine, solubilized with nonionic
20   detergent, and applied to a P-selectin affinity
column. After extensive washing, bound material
was eluted with EDTA and analyzed by SDS-PAGE
followed by fluorography. Samples were
electrophoresed on 10% SDS polyacrylamide gels
25   under both nonreducing and reducing conditions and
analyzed by fluorography. Other samples were
either sham treated or digested with 1 U/ml of
sialidase for 24 h or with 3.3 U/ml of PNGaseF for
24 h, and then electrophoresed on 10% SDS
30   polyacrylamide gels under reducing conditions and
analyzed by fluorography.

A single metabolically labeled species was
eluted, which co-migrated under both nonreducing
and reducing conditions with the major species
35   detected in neutrophil and HL-60 cell membranes by
blotting with [^{125}I]P-selectin. Only 0.15-0.5% of
the total [^{3}H]glucosamine-labeled HL-60

26

glycoproteins bound to the P-selectin column,
indicating that the ligand is not abundant.
Sialidase treatment of the [³H]glucosamine-labeled P
selectin ligand from HL-60 cells produced the same

5   increase in apparent molecular weight that was
observed for the major neutrophil ligand identified
by the P-selectin blotting assay. In addition,
PNGaseF treatment caused the same decrease in the
apparent molecular weight of the HL-60 cell ligand

10   that was observed for the neutrophil ligand.

        Comparison of the P-selectin Ligand with Known
        Neutrophil Membrane Proteins

        The properties of the major 120,000 D P-selectin
        ligand were compared with those of three well-

15   characterized neutrophil membrane proteins with
        similar apparent molecular weight. The first two
        molecules, lamp-1 and lamp-2, are abundant
        neutrophil proteins that are predominantly
        localized in lysosomal membranes but are also

20   expressed in small amounts on the cell surface.
        These proteins have a large number of complex N-
        linked glycan chains, many of which carry the
        sialyl Le^x tetrasaccharide. Polyclonal antisera
        (1:5 dilution) and MAbs (40 µg/ml) to lamp-1 (CR3)

25   and lamp-2 (BB6) had no effect on binding of P-
        selectin to neutrophils as assessed by flow
        cytometry.

        Membrane extracts (200 µg protein/lane) were
        electrophoresed on 7.5% SDS-polyacrylamide gels

30   under nonreducing or reducing conditions,
        transferred to Immobilon™ membranes, and probed
        with [¹²⁵I]P-selectin or murine monoclonal antibodies
        directed against human lamp-1 (CR3), human lamp-2
        (BB6), human L-selectin (DREG-200), or human

35   leukosialin (Leu22). Western blot analysis of
        neutrophil membranes with MAbs to lamp-1 and lamp-2
        showed that the electrophoretic mobilities of these

27

proteins under nonreducing conditions were distinct
from that of the P-selectin ligand. In contrast to
the P-selectin ligand, the electrophoretic
mobilities of lamp-1 and lamp-2 are not affected by
5   sialidase treatment. Although lamp-1 and lamp-2
from myeloid cells are rich in lactosaminoglycans
sensitive to endo-β-galactosidase, treatment of
intact neutrophils with the enzyme did not affect
binding of [^{125}I]P-selectin. Pretreatment of crude
10 neutrophil membrane extracts or WGA column eluate
with endo β-galactosidase (200 mU/ml, 1-2 h, 37°C)
also did not affect the apparent molecular weight
of the ligand or its ability to bind [^{125}I]P-
selectin. These data argue that lamp-1 and lamp-2
15 are not ligands for P-selectin even though they
carry many sialyl Le^x structures.

       The third molecule whose apparent molecular
weight is similar to the 120,000 D P-selectin
ligand is CD43 (leukosialin, sialophorin), a
20 heavily sialylated membrane protein present on
platelets and all leukocytes. It carries numerous
O-linked sugar chains and is differentially
glycosylated by cells of various hematopoietic
lineages. Like the P-selectin ligand, treatment of
25 leukosialin with sialidase increases its apparent
molecular weight. However, in contrast to the P-
selectin ligand, the electrophoretic mobility of
leukosialin was unaffected by reduction.
Monospecific polyclonal anti-human leukosialin
30 antisera (1:5 dilution) did not inhibit P-selectin
binding to neutrophils as assessed by flow
cytometry. Furthermore, immunodepletion of
leukosialin from neutrophil membrane extracts did
not deplete P-selectin ligand as assessed by the
35 blotting assay. Finally, leukosialin purified from
HL-60 cells did not bind P-selectin. Neutrophil
WGA eluate (50 μg) and leukosialin purified from

28

HL-60 cells (0.5 μg) were electrophoresed under
reducing conditions on 7.5% SDS-polyacrylamide
gels, transferred to Immobilon™, and probed with
[^{125}I]P-selectin. The same membrane was then probed
5   with the monoclonal anti-human leukosialin antibody
Leu22.

Based on studies in which an antibody to L-
selectin (DREG-56) partially inhibited neutrophil
adhesion to P-selectin-transfected cells, it was
10   suggested that L-selectin is an important
glycoprotein ligand on myeloid cells for P-selectin
by Picker et al., *Cell*, *66*: 921-933 (1991).
Although L-selectin is present in membrane extracts
and WGA eluates of neutrophil membranes, as
15   detected by Western blotting, [^{125}I]P-selectin did
not bind to L-selectin in the blotting assay. In
addition, the anti-L-selectin MAb DREG-56 (100
μg/ml) had no effect on the binding of purified P-
selectin to quiescent neutrophils as assessed by
20   flow cytometry. Neutrophils were preincubated for
15 min with buffer alone, 100 μg/ml of the anti-L-
selectin monoclonal antibody DREG-56, or 100 μg/ml
of the anti-P-selectin MAb G1 before addition of
buffer or P-selectin. P-selectin binding was then
25   detected by sequential incubation of the cells with
biotinylated S12 (a noninhibitory monoclonal
antibody to P-selectin) and phycoerythrin-
streptavidin as described in Materials and Methods.
Parallel control assays showed that the
30   neutrophils expressed high levels of L-selectin
detectable by DREG-56. Binding of the anti-L-
selectin MAb DREG-56 to the neutrophils was
assessed by indirect immunofluorescence using a
phycoerythrin-conjugated anti-murine IgG$_1$ antibody.
35   Identical results were obtained with the anti-L-
selectin MAbs DREG-55 and DREG-200. Thus,
interactions with L-selectin do not appear to

29

contribute to the binding of fluid-phase P-selectin
to intact neutrophils or to immobilized proteins
from neutrophil membrane extracts.

The following additional determinations and
5   observations have been made relating to the
glycoprotein ligand for P-selectin.
        Amino Acid Sequences of Peptides of the
        P-selectin Ligand from Human Neutrophils
        Amino acid sequence data on tryptic peptides
10   derived from the sample sent to Harvard Microchem
revealed two peptides that did not match any known
sequence in the Brookhaven Protein Data Bank,
SWISS-PROT or PIR protein sequence databases, or
the translated GENBANK database. These two
15   peptides HMYPVR (Sequence I.D. No. 1) and PGLTPEP
(Sequence I.D. No. 2) correspond to amino acids
340-345 and amino acids 380-386 respectively of a
cDNA cloned from an HL-60 cDNA library, reported by
T. M. Veldmann of Genetics Institute at the meeting
20   on "Cell Adhesion: Regulation and Clinical
Prospects" in Amsterdam on October 15, 1993. Based
on limited functional and structural data presented
by Dr. Veldmann, it appears likely that this cDNA
represents the P-selectin ligand.
25   Demonstration that the 120 kD P-selectin Ligand
        from Human Neutrophils Contains N-linked
        Oligosaccharides That Are Not Required for
        P-selectin Binding
        Studies described above demonstrated that the P-
30   selectin ligand contains a limited number of N-
linked glycan chains and that enzymatic removal of
these chains with PNGaseF did not affect the
ability of the ligand to bind [^{125}I]P-selectin using
the P-selectin blotting assay. To address this
35   question using an independent and more quantitative
approach [^{125}I]P-selectin ligand was digested with

PNGaseF either with or without prior denaturation
with SDS. As before, the PNGaseF digestion
decreased the apparent molecular weight of the
ligand as assessed by SDS-PAGE and autoradiography.
5   In addition, enzyme treatment abolished the ability
of [^{125}I]P-selectin ligand to bind to concanavilin A,
a plant lectin which binds to the tri-mannose core
characteristic of all N-linked glycans. This
indicated that the enzyme effectively removed N-
10   linked chains from the ligand. However, the
binding of [^{125}I]P-selectin ligand to immobilized P-
selectin was unaffected by PNGaseF treatment.
       Demonstration that the 120 kD P-selectin Ligand
       from HL-60 cells and Human Neutrophils Contains
15   O-linked Oligosaccharides
       The nature of the oligosaccharides on the 120 kD
glycoprotein P-selectin ligand from both HL-60
cells and neutrophils for P-selectin was examined.
HL-60 cells were cultured in media containing
20   radioactive [6-^{3}H]glucosamine, as described above.
This precursor is efficiently converted by cells to
radioactive GlcNAc, GalNAc, and sialic acid. The
ligand was then purified by affinity chromatography
on a column of P-selectin-Affigel 15™ and the
25   radiolabeled material was digested with the
commercial protease preparation called Pronase. The
Pronase-derived glycopeptides were treated with A.
ureafaciens neuraminidase which released
approximately 25% of the radioactivity as N-
30   acetylneuraminic acid. The residual glycopeptides
were then hydrolyzed in strong acid (2 N HCl for 4
h at 100°C and the hydrolyzed material (minus the
sialic acid which is destroyed by this treatment)
was analyzed by both high performance anion
35   exchange chromatography on a PA-1 Dionex column and
by descending paper chromatography of the material
after reacetylation by treatment with acetic

31

anhydride according to standard procedures. The
remaining radioactivity in the glycopeptides was
composed of N-acetylgalactosamine (GalNAc) and N-
acetylglucosamine (GlcNAc) in the approximate ratio

5   of 1:2, respectively. This indicates that the
overall composition of amino sugars in the 120 kD
P-selectin ligand is approximately 25% sialic acid,
50% GlcNAc and 25% GalNAc. The P-selectin ligand
purified from human neutrophils was analyzed by

10   Dionex HPAE chromatography following neuraminidase
treatment and all sialic acid was recovered as N-
acetylneuraminic acid (see also, Norgard et al.,
1993). One microgram of the purified human
neutrophil ligand of 120 kD was hydrolyzed with 2 N

15   trifluoroacetic acid according to standard
procedures, and the released monosaccharides
(except for sialic acid which is destroyed by this
treatment) were analyzed by high performance anion
exchange chromatography on a PA-1 Dionex column and

20   detected by pulsed amperometric detection. The
results indicated that the sample contained 233
picomoles of fucose, 70 picomoles of $GalNH_2$
(recorded as GalNAc), and approximately 200
picomoles of $GlcNH_2$ (recorded as GlcNAc). On a molar

25   basis, assuming the molecular weight of the ligand
to be in the range of 100,000 for sake of argument,
then 1 microgram is approximately 10 picomoles of
glycoprotein. This suggests that each mole of
ligand contains approximately 23 moles of fucose, 7

30   moles of GalNAc and 20 moles of GlcNAc.
        The P-selectin ligand from HL-60 cells was
purified from cells grown in media containing
either [2-^{3}H]mannose or [^{14}C]fucose. These
precursors allow specific radiolabeling of mannose

35   and fucose residues, respectively. Both
radioactive mannose and fucose were recovered in
the purified P-selectin ligand, confirming that it,

like the neutrophil ligand, contains both mannose
and fucose.   Furthermore, when the ³H-fucose-labeled
ligand is treated with mild base and sodium
borohydride to effect beta-elimination, ³H-fucose-
5   labeled oligosaccharides are released that are both
high molecular weight and moderate molecular
weight, as estimated by chromatography on a column
of BioGel™ P-10.

        The presence of large amounts of GalNAc in the
10   P-selectin ligand suggested that the protein might
contain large amounts of Ser/Thr-linked (O-linked)
oligosaccharides.   To test for the presence of this
linkage, three types of experiments were performed.
The presence of O-linked oligosaccharide was
15   established by the observation that the ligand
binds quantitatively to Jacalin-Sepharose, an
immobilized plant lectin that binds to the core
disaccharide sequence Galß1-3GalNAcα-Ser/Thr in
glycoproteins.   Jacalin-Sepharose can bind to O-
20   linked oligosaccharides that have modifications of
this simple core.   Thus, these results are not in
conflict with the lack of sensitivity of the ligand
to O-glycanase as outlined below.

        Simple, nonsialylated O-linked oligosaccharides
25   with the sequence Galß1-3GalNAcα-Ser/Thr are
cleaved by the endoglycosidase, O-Glycanase
(Genzyme).   Treatment of the ³H-glucosamine-labeled
P-selectin ligand from HL-60 cells with
neuraminidase to remove sialic acids from N- and O-
30   linked glycans caused a decrease in electrophoretic
mobility of the ligand when analyzed by SDS-PAGE,
as described previously.   However, subsequent
treatment of the ligand with O-glycanase caused no
additional change in mobility.   This suggests that
35   the O-linked oligosaccharides on the P-selectin
ligand have large, complex structures that are not
susceptible to cleavage with O-glycanase.

In a second approach, [^{3}H]glucosamine-labeled P-
selectin ligand was purified from HL-60 cells and
subjected to treatment with mild base (0.05 M NaOH)
in the presence of sodium borohydride (1 M) for 16

5   h at 45°C. This condition effects the β-
elimination reaction and release of the intact O-
linked oligosaccharides with conversion of the
linking GalNAc to N-acetylgalactosaminitol. This
material was analyzed by gel filtration on a column

10   of BioGel P-10™, which separates oligosaccharides
primarily on the basis of their size. Such
techniques for the structural analysis of a mucin-
like glycoprotein from metabolically-radiolabeled
cells have previously been used in the analysis of

15   the LDL-receptor and more recently on studies of
the human transferrin receptor (Cummings, R.D.,
Kornfeld, S., Schneider, W.J., Hobgood, K.K.,
Tolleshaug, J., Brown, M.S., and Goldstein, J.L.,
J. Biol. Chem., *258*: 15261-15273 (1983); Do, S.-

20   I., and Cummings, R.D., *Glycobiology*, 2: 345-353
(1992), incorporated herein by reference. Before
the β-elimination reaction all of the radiolabeled
P-selectin ligand eluted in the void volume of the
column, as expected, indicating that the intact

25   material is large in size. After the β-elimination
reaction most of the radioactivity was included in
the column eluting in a size range of 1,000 to
1,500 daltons. This is a typical elution position
for sialylated O-linked oligosaccharides. The

30   unreleased N-linked oligosaccharides (now contained
on a base-hydrolyzed peptide) elute in a peak near
the void volume. The β-elimination reaction was
also performed on the 120 kD glycoprotein ligand
derived from human neutrophils. In that case the

35   ligand was post-radiolabeled on its sialic acid by
periodate oxidation followed by reduction with
NaB$_3$H$_4$. The β-eliminated material from the

34

neutrophil ligand eluted in a similar position on
the BioGel™ P-10 column. These results indicate
that both the HL-60 and human neutrophil derived
120 kD ligand for P-selectin contain large amounts

5   of O-linked oligosaccharides.

In a third approach to study the presence of O-
linked oligosaccharides on the P-selectin ligand,
the specificity of a novel protease from
Pasteurella hemolytica termed O-sialoglycoprotease

10   was exploited. This enzyme cleaves the peptide
backbone of proteins containing relatively
"clustered" sialylated Ser/Thr-linked
oligosaccharides (Norgard, et al., *J. Biol. Chem.*,
268: 12764-12774 (1993); Sutherland, D.R., et al.,

15   *J. Immunol.*, *148,* 1458-1464 (1992). The
[^{3}H]glucosamine-labeled P-selectin ligand was
purified as described above from HL-60 cells and
from human neutrophils. The latter were
radiolabeled as above with periodate/NaB$_3$H$_4$

20   treatment. The radiolabeled glycoproteins were
analyzed by SDS-PAGE and fluorography before and
after treatment with the O-sialoglycoprotease.
Treatment with the O-sialoglycoprotease caused
extensive degradation of the ligand. Interestingly,

25   treatment of intact HL-60 cells with the O-
sialoglycoprotease abolished their interaction with
purified membrane P-selectin, as evidenced by flow
cytometric analysis and cell adhesion to
immobilized P-selectin (Norgard, et al. *J. Biol.*

30   *Chem.*, *268*: 12764-12774 (1993)). In contrast, the
expression of the SLe^x antigen on the cells, as
evidenced by their binding to CSLEX1 MAb, was not
significantly affected by this protease treatment.
These results demonstrate that the 120 kD P-

35   selectin ligand from both human neutrophils and HL-
60 cells are glycosylated similarly and both have
abundant levels of O-linked oligosaccharides.

35

Evidence that the 120 kD P-selectin Ligand
Contains SLe^x Antigen

Three alternative approaches were used to show
that the 120 kD P-selectin ligand contains sLe^x. In

5   the first approach, the ^{3}H-glucosamine-labeled P-
selectin ligand from HL-60 cells was reapplied to a
column of P-selectin-Affigel™. When this
chromatography was done in the presence of antibody
to the SLe^x antigen (CSLEX1 monoclonal antibody, see

10 Fukushima, et al., *Cancer Res.*, 44: 5279-5285
(1984), purchased from Dr. Paul Teraski, University
of California, Los Angeles) binding was more than
90% reduced. In contrast, when a control
experiment was done in which the rechromatography

15 occurred in the presence of antibody to the Le^x
antigen, which lacks sialic acid, there was little
if any effect.

In an alternative approach, the 120 kD
glycoprotein ligand from human neutrophils was

20 purified by affinity chromatography on a column of
immobilized soluble truncated P-selectin (tPS)
(Ushiyama et. al. *J. Biol. Chem.*, 268: 15229-15237
(1993)). The material was analyzed by SDS-PAGE in
reducing conditions, transferred to Immobilon™

25 membrane, and probed for its reactivity with
radioiodinated P-selectin and the monoclonal
antibody CSLEX-1, which reacts with SLe^x. The 120
kD glycoprotein eluted from the P-selectin affinity
column reacts with ^{125}I-P-selectin. The same

30 material reacts with antibody to SLe^x, using the
technique of Western blotting. These results
demonstrate that the neutrophil-derived 120 kD
glycoprotein ligand for P-selectin contains SLe^x.

As another approach to this problem, the ^{125}I-

35 labeled form of the neutrophil-derived 120 kD
glycoprotein ligand for P-selectin, prepared as
described above, was analyzed for its ability to

adhere to wells of microtiter plates containing
immobilized CSLEX-1 (reactive with SLe^x epitopes)
and LeuM1 antibody (reactive with Le^x epitopes).
The radiolabeled ligand bound to both immobilized

5   antibodies, as well as to immobilized P-selectin,
as expected. Reactivity with immobilized CSLEX-1
was destroyed by *A. ureafaciens* neuraminidase
treatment of the ligand. Reactivity to the LeuM-1
was destroyed by treatment of the radioiodinated

10 ligand with the *Streptomyces sp.* α1,3/4 fucosidase.
Fucosidase treatment did not affect binding to P-
selectin. This is not surprising, since other data
indicated that the *Streptomyces sp.* α1,3/4
fucosidase cannot release fucose from sialylated

15 oligosaccharides containing SLe^x epitopes.
Together, these results strongly indicate that the
ligand contains SLe^x epitopes.

    Evidence that the P-selectin Ligand Contains
    Poly-N-acetyllactosamine Sequences

20   Treatment of the radioiodinated ligand with *E.*
freundii endo-β-galactosidase abolished binding to
LeuM-1 antibody. This enzyme is an endoglycosidase
that cleaves certain oligosaccharides containing
the repeating unit [→3Galβ1→4GlcNAcβ1→]$_n$, where n ≥

25 2, at internal β-galactosyl residues. These chains
constitute the so-called poly-N-acetyllactosamine
sequence (or polylactosaminoglycan). The results
demonstrate that the P-selectin contains
polylactosaminoglycan sequences and that these are

30 fucosylated. The ^{3}H-glucosamine-labeled P-selectin
ligand from HL-60 cells quantitatively binds to a
column of immobilized tomato lectin, a plant lectin
which has been shown to bind to poly-N-
acetyllactosamine sequences within glycoproteins.

35 In addition, it should be noted that these
polyfucosylated and sialylated
polylactosaminoglycans are not highly sensitive to

endo-β-galactosidase because of the terminal sialic
acids. This explains why neither the binding of
the radioiodinated neutrophil-derived P-selectin
ligand to immobilized P-selectin nor to immobilized
5   CSLEX-1 antibody is abolished by treatment with
endo-β-galactosidase.

    <u>Demonstration that the 120 kD P-selectin Ligand</u>
    <u>from HL-60 cells is Sulfated</u>

    HL-60 cells were metabolically-radiolabeled with
10   Na^{35}SO$_4$ to examine whether the 120 kD ligand for P-
selectin is sulfated. Approximately 2 x 10^6
cells/ml were grown in media containing 0.15 mCi/ml
of Na^{35}SO$_4$ for 48 h in RPMI supplemented with 10%
fetal bovine serum, 2 mM glutamine, 100 IU/ml
15   penicillin and 100 μg/ml streptomycin. The P-
selectin glycoprotein ligand was then purified as
described above. Fractions (1 ml) from the
P-selectin-immobilized affinity column were
collected and radioactivity monitored by liquid
20   scintillation counting. The metabolically-
radiolabeled glycoprotein eluted from the P-
selectin column was precipitated by addition of
ice-cold trichloroacetic acid (10% final). The
pellets were washed with 1 ml of cold acetone, 0.2%
25   HCl, resuspended in Laemmli sample buffer, and
analyzed by SDS-PAGE in 7.5% acrylamide. The gel
was processed for fluorography with EN^{3}HANCE™
according to the manufacturer's instructions. The
dried gel was exposed to Fuji RX film at -80°C for
30   7 days. The results indicated that the 120 kD
glycoprotein bound by P-selectin is radiolabeled by
^{35}SO$_4$.

    The differential mobility of the major ligand
during SDS-PAGE in the presence and absence of
35   reducing agents indicates that the native ligand is
a disulfide-linked homodimer. A homodimeric ligand
with two equivalent binding sites might enhance the

38

avidity of the interaction with P-selectin. The
ability of [^{125}I]P-selectin to bind to the ligand
after reduction and denaturation with SDS suggests
that higher order structural features of the
5   protein are not critical for recognition.

The blotting assay also detected two minor
ligands. The first has an approximately 250,000 M_r
under reducing conditions. Because its mobility is
identical to that of the major ligand under
10 nonreducing conditions, it may represent a
subpopulation of the major ligand that is resistant
to reduction. The second has an approximately
160,000 M_r under nonreducing conditions. Binding of
P-selectin to both minor ligands was Ca^{2+}-dependent
15 and blocked by the MAb G1.

The isolation of a single glycoprotein from
metabolically labeled HL-60 cells suggests that P-
selectin has a marked preference for a particular
ligand structure. L-selectin, which is expressed
20 on leukocytes and binds to sialylated structures on
endothelial cells, interacts preferentially with
50,000 D and 90,000 D sulfated, fucosylated
glycoproteins from murine peripheral lymph nodes
(Imai, et al., *J. Cell Biol.*, *113*: 1213-1222
25 (1991)). Thus, both P-selectin and L-selectin
appear to interact with a small subset of
glycoprotein ligands.

It has been demonstrated that L-selectin on
neutrophils carries the sialyl Le^x epitope and that
30 a MAb to L-selectin partially blocks neutrophil
adhesion to cells transfected with P-selectin cDNA
(Picker, et al., *Cell*, *66*: 921-933 (1991)). Based
on these observations, it was proposed that L-
selectin on neutrophils is a predominant ligand for
35 P-selectin. However, no direct interaction of L-
selectin with P-selectin was demonstrated. Binding
of P-selectin to L-selectin in neutrophil membrane

extracts was not detectable. Furthermore, the
binding of P-selectin to intact neutrophils is
unaltered by antibodies to L-selectin or by
neutrophil activation that causes shedding of L-
5   selectin from the cell surface. Although it is
conceivable that L-selectin has weak affinity for
P-selectin, the significance of this potential
interaction remains to be established.

A recombinant P-selectin IgG chimera was shown
10   to bind to myeloid cells and to a sulfatide, Gal(3-
SO_4) ß1-Ceramide by Aruffo et al., *Cell*, *67*: 35-44
(1991). Sulfatide also inhibited interaction of
the chimera with monocytoid U937 cells, as reported
by Aruffo et al., (1991). It was not demonstrated
15   whether binding of the P-selectin chimera to the
cells or to sulfatide was Ca^{2+} dependent, a
fundamental characteristic of selectin-dependent
cellular interactions. Protease digestion of
intact cells should increase the accessibility of
20   P-selectin to potential glycolipid ligands such as
sulfatides. However, protease treatment abolishes
binding of P-selectin to neutrophils and HL-60
cells as well as adhesion of neutrophils to
immobilized P-selectin. In addition, although
25   erythrocytes and platelets express sulfatides, they
do not specifically interact with P-selectin.
Thus, it seems unlikely that sulfatides are the
principal mediators of adhesion of myeloid cells to
P-selectin. It remains to be determined whether
30   sulfatides inhibit binding of P-selectin to myeloid
cells by specific competition with a glycoprotein
ligand or by indirect effects. Because the P-
selectin ligand described herein is sulfated, it
may contain structural features that are mimicked
35   by sulfatides.

Previous studies by Zhou et al., (1991); and
Polley et al., (1991) have shown that P-selectin

interacts with α(2-3) sialylated, α(1-3)fucosylated
lactosaminoglycans, of which one is the sialyl Le^x
tetrasaccharide. However, several observations
suggest that the sialyl Le^x tetrasaccharide *per se*

5   does not bind with high affinity to P-selectin.
First, some investigators (Moore et al., (1991);
Aruffo et al., (1991); Polley, et al., (1991)), but
not all, have found that sialyl Le^x inhibits
interactions of myeloid cells with P-selectin.

10   Second, CHO cells transfected with a
fucosyltransferase express sialyl Le^x yet bind P-
selectin with significantly lower affinity than do
myeloid cells (Zhou et al., (1991)). Third, HT-29
cells, which also express sialyl Le^x, do not

15   interact at all with P-selectin (Zhou et al.,
1991). Finally, several neutrophil membrane
proteins known to carry the sialyl Le^x structure,
are distinct from the major glycoprotein ligand
identified herein and do not bind P-selectin in the

20   assays described here. These observations suggest
that the ligand contains structural features in
addition to the sialyl Le^x tetrasaccharide that
enhance the affinity and/or specificity of its
interaction with P-selectin.

25   A blotting assay of neutrophil and HL-60 cell
membrane extracts was used to search for ligands
for P-selectin. As described previously in WO
92/01718, [^{125}I]P-selectin bound preferentially to a
glycoprotein of Mr 120,000 as assessed by SDS-PAGE

30   under reducing conditions. Under nonreducing
conditions, the ligand for P-selectin had an
apparent Mr of 250,000, suggesting that it is a
disulfide-linked homodimer. In initial studies,
the ligand was partially purified by serial

35   affinity chromatography on wheat germ agglutinin
(WGA) and P-selectin affinity columns. Proteins
bound to the P-selectin column were eluted with

EDTA. The glycoprotein ligand was greatly enriched
in the EDTA eluate from the P-selectin column, as
assessed by the intensity of the band identified by
[^{125}I]P-selectin blotting. As noted in WO 92/01718,
5   however, the ligand stained poorly with silver,
consistent with its being an unusually heavily
glycosylated protein. In the initial
purifications, the only contaminating protein
present noted by silver staining of the gel was a
10   small amount of P-selectin itself which had been
leached from the affinity column. Using a new P-
selectin affinity column and more extensive washing
procedures documented in the methods, the ligand
has now been isolated free from contaminants. This
15   conclusion is based on observation that there are
no silver staining bands present but the ligand is
clearly identified by its ability to interact with
[^{125}I]P-selectin in the blotting assay.

As described in WO 92/01718, partial removal of
20   sialic acids with sialidase slowed the mobility of
the ligand, a feature characteristic of heavily
sialylated glycoproteins. Extensive sialidase
digestion abolished recognition of the ligand by P-
selectin. It has now been demonstrated that the
25   ligand contains both N- and O-linked
oligosaccharides. Further, the material was shown
to be pure as assessed by SDS-PAGE and
autoradiography following radioiodination.

A form of the ligand in which the carbohydrate
30   components are radiolabeled has also been purified
by P-selectin affinity chromatography, as described
above. SDS-PAGE analysis of the P-selectin column
eluate, followed by fluorography, indicates that
the only labeled protein has an Mr of 250,000 under
35   nonreducing conditions and 120,000 under reducing
conditions. The radiolabeled ligand has the same
shifts in electrophoretic mobility following

treatment with sialidase or PNGase F. Thus, all
the features of the radiolabeled ligand correspond
to those of the ligand identified by the P-selectin
blotting assay. Because only a single radiolabeled
5   species is isolated from the P-selectin affinity
column, the carbohydrate structures of the ligand
can be analyzed in detail by procedures that have
been developed, for example, as reported by R.D.
Cummings and S. Kornfeld, *J. Biol. Chem.*, *257*:
10   11235-11240 (1982) and R.D. Cummings, et al., *J.*
Biol. Chem., *258*: 15261-15273 (1983).

In summary, the glycoprotein ligand for P-
selectin from myeloid cells has the characteristics
of a disulfide-linked homodimer with each subunit
15   having an apparent Mr of 120,000 as assessed by
SDS-PAGE. The protein has some N-linked
carbohydrate but its most striking feature is the
presence of a large number of clustered sialylated
O-linked glycans, most of which appear to be larger
20   than the usual simple O-linked chains cleaved by O-
glycanase. Although the ligand contains the sLe^x
structure, the data indicate that additional
structural features in the ligand are required to
confer high affinity binding to P-selectin. These
25   features include, but are not limited to,
carbohydrate structures of more complexity than sLe^x
itself including difucosyl sialyl Le^x and longer
polyfucosylated polylactosaminoglycans, clustering
of many glycan chains to increase avidity, and
30   specific orientations of the glycans relative to
the protein backbone.

Preparation of Diagnostic and Therapeutic Agents
Derived from the Protein or Carbohydrate
Components of the Glycoprotein Ligand for P-
selectin.

5   The glycoprotein ligand for P-selectin described
above has a variety of applications as diagnostic
reagents and, potentially, in the treatment of
numerous inflammatory and thrombotic disorders.

Diagnostic Reagents

10   Antibodies to the ligand, fragments thereof, or
its carbohydrate or polypeptide components, can be
prepared by methods known in the art (e.g., Harlow,
E. and Lane, D., in Antibodies: A Laboratory Manual
(Cold Spring Harbor Laboratory Press, Cold Spring

15   Harbor, NY, 1988). Such antibodies can be used for
the detection of human disorders in which P-
selectin ligands might be defective. Such
disorders would most likely be seen in patients
with increased susceptibility to infections in

20   which leukocytes might not be able to bind to
activated platelets or endothelium. Cells to be
tested, usually leukocytes, are collected by
standard medically approved techniques and
screened. Detection systems include ELISA

25   procedures, binding of radiolabeled antibody to
immobilized activated cells, flow cytometry,
immunoperoxidase or immunogold analysis, or other
methods known to those skilled in the arts.

       Antibodies directed specifically to protein or

30   carbohydrate components of the ligand can be used
to distinguish defects in expression of the core
protein or in glycosyltransferases and/or modifying
enzymes that construct the proper oligosaccharide
chains on the protein. The antibodies can also be

35   used to screen cells and tissues other than
leukocytes for expression of the protein or

44

carbohydrate components of the ligand for P-
selectin.

The identification of amino acid sequences which
are specific for the P-selectin ligand enables one
5   to design and synthesize nucleic acid probes for
use in cloning and detecting nucleic acid
sequences, e.g., in genomic or cDNA libraries,
encoding the polypeptide component of the P-
selectin ligand. For example, because both of the
10   sequences, HMYPVR and PGLTPEP, are unique to the
ligand and relatively short, degenerate nucleic
acid probes can be synthesized which possess all
possible codons for each amino acid sequence, using
standard oligonucleotide synthetic methods (see,
15   e.g., Sambrook et al., In Molecular Cloning: A
Laboratory Manual, second ed., Chapter 11, pp.
11.1-11.61 (Cold Spring Harbor Laboratory Press,
Cold Spring Harbor, 1989)). Such synthetic probes
can be labeled by any of a variety of methods and
20   used to screen human genomic and cDNA libraries for
nucleic acid molecules encoding the structural
coding sequence for the P-selectin ligand (see,
e.g., Sambrook et al., 1989). Alternatively,
antibodies directed against the ligand, or its
25   carbohydrate or polypeptide components, can also be
used to screen cDNA libraries for expression of
ligand protein to identify clones containing
nucleic acid molecules encoding the ligand.

       Complementary DNA clones encoding the
30   polypeptide component of the ligand can be isolated
and sequenced. These probes can be used as
diagnostic reagents to examine expression of RNA
transcripts for the ligand in leukocytes and other
tissues by standard procedures such as Northern
35   blotting of RNA isolated from cells and in situ
hybridization of tissue sections.

45

A similar approach can be used to determine qualitative or quantitative disorders of P-selectin itself. The glycoprotein ligand, carbohydrates, or appropriate derivatives thereof, is labeled and

5   tested for its ability to bind to P-selectin on activated platelets from patients with disorders in which P-selectin might be defective.

The ligand, or components thereof, can also be used in assays of P-selectin binding to screen for

10   compounds that block interactions of P-selectin with the ligand.

        Clinical Applications

        Since P-selectin has several functions related to leukocyte adherence, inflammation, tumor

15   metastases, and coagulation, clinically, compounds which interfere with binding of P-selectin and/or the other selectins, including E-selectin and L-selectin, such as the carbohydrates, can be used to modulate these responses. These compounds include

20   the P-selectin ligand, antibodies to the ligand, and fragments thereof. For example, the glycoprotein ligand, or components thereof, particularly the carbohydrate moieties, can be used to inhibit leukocyte adhesion by competitively

25   binding to P-selectin expressed on the surface of activated platelets or endothelial cells. Similarly, antibodies to the ligand can be used to block cell adhesion mediated by P-selectin by competitively binding to the P-selectin ligand on

30   leukocytes or other cells. These therapies are useful in acute situations where effective, but transient, inhibition of leukocyte-mediated inflammation is desirable. In addition, treatment of chronic disorders may be attained by sustained

35   administration of agents, for example, by subcutaneous or oral administration.

46

An inflammatory response may cause damage to the host if unchecked, because leukocytes release many toxic molecules that can damage normal tissues. These molecules include proteolytic enzymes and
5   free radicals. Examples of pathological situations in which leukocytes can cause tissue damage include injury from ischemia and reperfusion, bacterial sepsis and disseminated intravascular coagulation, adult respiratory distress syndrome, tumor
10   metastasis, rheumatoid arthritis and atherosclerosis.

Reperfusion injury is a major problem in clinical cardiology. Therapeutic agents that reduce leukocyte adherence in ischemic myocardium
15   can significantly enhance the therapeutic efficacy of thrombolytic agents. Thrombolytic therapy with agents such as tissue plasminogen activator or streptokinase can relieve coronary artery obstruction in many patients with severe myocardial
20   ischemia prior to irreversible myocardial cell death. However, many such patients still suffer myocardial neurosis despite restoration of blood flow. This "reperfusion injury" is known to be associated with adherence of leukocytes to vascular
25   endothelium in the ischemic zone, presumably in part because of activation of platelets and endothelium by thrombin and cytokines that makes them adhesive for leukocytes (Romson et al., Circulation, 67: 1016-1023 (1983)). These adherent
30   leukocytes can migrate through the endothelium and destroy ischemic myocardium just as it is being rescued by restoration of blood flow.

There are a number of other common clinical disorders in which ischemia and reperfusion results
35   in organ injury mediated by adherence of leukocytes to vascular surfaces, including strokes; mesenteric and peripheral vascular disease; organ

transplantation; and circulatory shock (in this
case many organs might be damaged following
restoration of blood flow).

Bacterial sepsis and disseminated intravascular
5   coagulation often exist concurrently in critically
ill patients. They are associated with generation
of thrombin, cytokines, and other inflammatory
mediators, activation of platelets and endothelium,
and adherence of leukocytes and aggregation of
10   platelets throughout the vascular system.
Leukocyte-dependent organ damage is an important
feature of these conditions.

Adult respiratory distress syndrome is a
devastating pulmonary disorder occurring in
15   patients with sepsis or following trauma, which is
associated with widespread adherence and
aggregation of leukocytes in the pulmonary
circulation. This leads to extravasation of large
amounts of plasma into the lungs and destruction of
20   lung tissue, both mediated in large part by
leukocyte products.

Two related pulmonary disorders that are often
fatal are in immunosuppressed patients undergoing
allogeneic bone marrow transplantation and in
25   cancer patients suffering from complications that
arise from generalized vascular leakage resulting
from treatment with interleukin-2 treated LAK cells
(lymphokine-activated lymphocytes). LAK cells are
known to adhere to vascular walls and release
30   products that are presumably toxic to endothelium.
Although the mechanism by which LAK cells adhere to
endothelium is not known, such cells could
potentially release molecules that activate
endothelium and then bind to endothelium by
35   mechanisms similar to those operative in
neutrophils.

48

Tumor cells from many malignancies, including
carcinomas, lymphomas, and sarcomas, can
metastasize to distant sites through the
vasculature. The mechanisms for adhesion of tumor
5   cells to endothelium and their subsequent migration
are not well understood, but may be similar to
those of leukocytes in at least some cases.
Specifically, certain carcinoma cells have been
demonstrated to bind to both E-selectin, as
10   reported by Rice and Bevilacqua, *Science, 246*:
1303-1306 (1991), and P-selectin, as reported by
Aruffo, et al., *Proc. Natl. Acad. Sci. USA, 89*:
2292-2296 (1992) and Stone and Wagner, *J. Clin.
Invest., 92*: 804-813 (1993). The association of
15   platelets with metastasizing tumor cells has been
well described, suggesting a role for platelets in
the spread of some cancers. Since P-selectin is
expressed on activated platelets, it is believed to
be involved in association of platelets with at
20   least some malignant tumors.

Platelet-leukocyte interactions are believed to
be important in atherosclerosis. Platelets might
have a role in recruitment of monocytes into
atherosclerotic plaques; the accumulation of
25   monocytes is known to be one of the earliest
detectable events during atherogenesis. Rupture of
a fully developed plaque may not only lead to
platelet deposition and activation and the
promotion of thrombus formation, but also the early
30   recruitment of neutrophils to an area of ischemia.

Another area of potential application is in the
treatment of rheumatoid arthritis.

In these clinical applications, the glycoprotein
ligand of P-selectin, comprising peptides having
35   the sequences HMYPVR and PGLTPEP, or fragments of
the ligand that retain P-selectin binding ability,
can be administered to block selectin-dependent

49

interactions by binding competitively to P-selectin
expressed on activated cells. In addition,
carbohydrate components of the ligand, which play a
key role in recognition by P-selectin, can be
5   administered alone, as well as attached to all or a
fragment of the polypeptide component of the
ligand. Similarly, natural or synthetic analogs of
the ligand or its fragments which bind to P-
selectin can also be administered to a patient to
10   block P-selectin dependent interactions. In
addition, antibodies to the polypeptide and/or
carbohydrate components of the ligand, or fragments
thereof, can be administered. The antibodies are
preferrably of human origin or modified to delete
15   those portions most likely to cause an immunogenic
reaction. The ligand, or fragments thereof,
carbohydrate components of the ligand, and
antibodies to the ligand molecule or its
carbohydrate or polypeptide components, in an
20   appropriate pharmaceutical carrier, are preferably
administered intravenously where immediate relief
is required. Other modes of administration include
intramuscularly, intraperitoneally, subcutaneously,
and orally. The carbohydrate component of the
25   ligand may also be conjugated to a carrier
molecule, or incorporated into a drug delivery
device for more effective and prolonged delivery to
a patient. The carbohydrate can also be modified
chemically to increase its *in vivo* half-life.
30   The carbohydrate can be isolated from cells
expressing the carbohydrate, either naturally or as
a result of genetic engineering as described in the
transfected COS cell examples, or, preferably, by
synthetic means. These methods are known to those
35   skilled in the art. In addition, a large number of
glycosyltransferases have been cloned (J.C. Paulson
and K.J. Colley, *J. Biol. Chem.*, *264*: 17615-17618

(1989)). Accordingly, workers skilled in the art
can use a combination of synthetic chemistry and
enzymatic synthesis to make pharmaceuticals or
diagnostic reagents.

5   The P-selectin glycoprotein ligand and protein
fragments of the ligand can also be administered as
a pharmaceutically acceptable acid- or base-
addition salt, formed by reaction with inorganic
acids such as hydrochloric acid, hydrobromic acid,

10   perchloric acid, nitric acid, thiocyanic acid,
sulfuric acid, and phosphoric acid, and organic
acids such as formic acid, acetic acid, propionic
acid, glycolic acid, lactic acid, pyruvic acid,
oxalic acid, malonic acid, succinic acid, maleic

15   acid, and fumaric acid, or by reaction with an
inorganic base such as sodium hydroxide, ammonium
hydroxide, potassium hydroxide, and organic bases
such as mono-, di-, trialkyl and aryl amines and
substituted ethanolamines.

20   Carbohydrate and polypeptide components and
fragments of the glycoprotein ligand of P-selectin
that are biologically active are those which, like
the full-length P-selectin glycoprotein ligand,
inhibit binding of leukocytes to P-selectin.

25   Suitable pharmaceutical vehicles for administration
to a patient are known to those skilled in the art.
For parenteral administration, a biologically
active carbohydrate or protein fragment of the P-
selectin glycoprotein ligand, or the entire

30   P-selectin ligand, will usually be dissolved or
suspended in sterile water or saline. For enteral
administration, a carbohydrate component of the P-
selectin glycoprotein ligand, the P-selectin
glycoprotein ligand, and fragments thereof, will be

35   incorporated alone, or in combination into an inert
carrier in tablet, liquid, or capsular form.
Suitable carriers may be starches or sugars and

include lubricants, flavorings, binders, and other
materials of the same nature. The carbohydrate,
ligand, or fragments thereof, can also be
administered locally at a wound or inflammatory
5   site by topical application of a solution or cream.
     Alternatively, a carbohydrate component of the
ligand, the ligand, or fragments thereof, may also
be administered in, on or as part of, liposomes or
microspheres (or microparticles). Methods for
10   preparing liposomes and microspheres for
administration to a patient are known to those
skilled in the art. U.S. Patent No. 4,789,734
describe methods for encapsulating biological
materials in liposomes. Essentially, the material
15   is dissolved in an aqueous solution, the
appropriate phospholipids and lipids added, along
with surfactants if required, and the material
dialyzed or sonicated, as necessary. A good review
of known methods is by G. Gregoriadis, Chapter 14.
20   "Liposomes", Drug Carriers in Biology and Medicine
pp. 287-341 (Academic Press, 1979). Microspheres
formed of polymers or proteins are well known to
those skilled in the art, and can be tailored for
passage through the gastrointestinal tract directly
25   into the bloodstream. Alternatively, the
carbohydrate components of the P-selectin ligand,
the ligand, or fragments thereof, can be
incorporated and the microspheres, or composite of
microspheres, implanted for slow release over a
30   period of time, ranging from days to months. See,
for example, U.S. Patent No. 4,906,474, 4,925,673,
and 3,625,214.
     The carbohydrates should be active when
administered parenterally in amounts above about 1
35   μg/kg of body weight. For treatment of most
inflammatory disorders, the dosage range will be
between 0.1 to 30 mg/kg of body weight. A dosage

of 70 mg/kg may be required for some of the
carbohydrates characterized in the examples.
The criteria for assessing response to
therapeutic modalities employing the P-selectin
5   glycoprotein ligand, fragments thereof,
carbohydrate components of the P-selectin
glycoprotein ligand, or antibodies to the ligand or
its carbohydrate or polypeptide components is
dictated by the specific physiological and
10   pathological condition of the patient and will
generally follow standard medical practices. For
example, the criteria for the effective dosage to
prevent extension of myocardial infarction would be
determined by one skilled in the art by looking at
15   marker enzymes of myocardial necrosis in the
plasma, by monitoring the electrocardiogram, vital
signs, and clinical response. For treatment of
acute respiratory distress syndrome, one would
examine improvements in arterial oxygen, resolution
20   of pulmonary infiltrates, and clinical improvement
as measured by lessened dyspnea and tachypnea. For
treatment of patients in shock (low blood
pressure), the effective dosage would be based on
the clinical response and specific measurements of
25   function of vital organs such as the liver and
kidney following restoration of blood pressure.
Neurologic function would be monitored in patients
with stroke. Specific tests are used to monitor
the functioning of transplanted organs; for
30   example, serum creatinine, urine flow, and serum
electrolytes in patients undergoing kidney
transplantation.

53

SEQUENCE LISTING

(1) GENERAL INFORMATION:

     (i) APPLICANT: Board of Regents of the
University of   Oklahoma

5   (ii) TITLE OF INVENTION: Glycoprotein Ligand
For P-Selectin   and Methods of
Use Thereof

     (iii) NUMBER OF SEQUENCES: 2

10   (iv) CORRESPONDENCE ADDRESS:
         (A) ADDRESSEE: Patrea L. Pabst
         (B) STREET: 1100 Peachtree Street, Suite
2800
         (C) CITY: Atlanta
15   (D) STATE: Ga
         (E) COUNTRY: USA
         (F) ZIP: 30309-4530

     (v) COMPUTER READABLE FORM:
         (A) MEDIUM TYPE: Floppy disk
20   (B) COMPUTER: IBM PC compatible
         (C) OPERATING SYSTEM: PC-DOS/MS-DOS
         (D) SOFTWARE: PatentIn Release #1.0,
Version #1.25

     (vi) CURRENT APPLICATION DATA:
25   (A) APPLICATION NUMBER:
         (B) FILING DATE:
         (C) CLASSIFICATION:

     (vii) PRIOR APPLICATION DATA:
         (A) APPLICATION NUMBER: US 07/650,484
30   (B) FILING DATE: 05-FEB-1991

     (vii) PRIOR APPLICATION DATA:
         (A) APPLICATION NUMBER: US 07/554,199
         (B) FILING DATE: 17-JUL-1990

     (vii) PRIOR APPLICATION DATA:
35   (A) APPLICATION NUMBER: US 07/320,408
         (B) FILING DATE: 08-MAR-1989

     (viii) ATTORNEY/AGENT INFORMATION:
         (A) NAME: Pabst, Patrea L.
         (B) REGISTRATION NUMBER: 31,284
40   (C) REFERENCE/DOCKET NUMBER:
OMRF110CIP(4)

     (ix) TELECOMMUNICATION INFORMATION:
         (A) TELEPHONE: (404)-815-6508
         (B) TELEFAX: (404-815-6555

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 6 amino acids
       (B) TYPE: amino acid
5   (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

10   (v) FRAGMENT TYPE: N-terminal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    His Met Tyr Pro Val Arg
    1   5

(2) INFORMATION FOR SEQ ID NO:2:

15   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 7 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

20   (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: N-terminal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25   Pro Gly Leu Thr Pro Glu Pro
    1   5

We claim:

1. A ligand for P-selectin comprising a fucosylated sialylated glycoprotein containing glucosamine and galactosamine in the approximate ratio of 2:1, N-linked oligosaccharides, O-linked oligosaccharides or Ser/Thr-linked oligosaccharides containing the core disaccharide sequence Galß1-3GalNAcα-Ser/Thr, sialyl Lewis x antigen NeuAcα2-3Galß1-4[Fucα1-3]GlcNAcß-R, and poly-N-acetyllactosamine sequences, wherein the ligand has an apparent relative molecular weight of 120,000 as assessed by SDS-PAGE under reducing conditions, an apparent molecular weight of 250,000 as assessed by SDS-PAGE under non-reducing conditions.

2. The ligand of claim 1 comprises the amino acid sequences HMYPVR (Sequence I.D. No. 1) and PGLTPEP (Sequence I.D. No. 2).

3. The ligand of claim 1 wherein the ligand is isolated from myeloid cell membranes of human neutrophils:
    solubilizing the membranes of the myeloid cells to form a membrane extract, binding the membrane extract to an affinity chromatography column containing immobilized P-selectin, eluting the ligand from the affinity column with a calcium chelating agent loading the pooled ligand.

4. The ligand of claim 3 wherein the eluted ligand is further purified by chromatography on a high performance liquid chromatography column.

5. The ligand of claim 1 in the form of a dimer of the 120,000 molecular weight glycoprotein having a relative molecular weight by SDS-PAGE of 250,000 under non-reducing conditions.

56

6.   The ligand of claim 1 wherein treatment with
O-glycanase and neuraminidase does not demonstrably
affect the apparent mobility of the ligand on SDS-
PAGE, any more than neuraminidase alone, such that
the ligand contains few simple oligosaccharides in
O-linkage to Ser/Thr residues.

7.   The ligand of claim 1 wherein the poly-N-
acetyllactosamine sequences are of the type
$[3Gal\beta1-4GlcNAc\beta1]_n$.

8.   The ligand of claim 1 wherein partial
removal of sialic acids with sialidase slows the
electrophoretic mobility of the ligand during SDS-
PAGE.

9.   The ligand of claim 8 wherein extensive
sialidase digestion abolishes recognition of the
ligand by P-selectin.

10.   The ligand of claim 1 wherein treatment of
the ligand with an enzyme that removes N-linked
glycans reduces the apparent Mr of the ligand on
SDS-polyacrylamide gels by approximately 3,000
without affecting its ability to be bound by P-
selectin.

11.   The protein component of a fucosylated
sialylated glycoprotein containing glucosamine and
galactosamine in the approximate ratio of 2:1, N-
linked oligosaccharides, O-linked oligosaccharides
or Ser/Thr-linked oligosaccharides containing the
core disaccharide sequence $Gal\beta1-3GalNAc\alpha-Ser/Thr$,
sialyl Lewis x antigen $NeuAc\alpha2-3Gal\beta1-4[Fuc\alpha1-3]GlcNAc\beta-R$, and poly-N-acetyllactosamine
sequences, wherein the ligand has an apparent
relative molecular weight of 120,000 as assessed by

57

SDS-PAGE under reducing conditions and an apparent
molecular weight of 250,000 as assessed by SDS-PAGE
under non-reducing conditions.

12. The protein of claim 11 comprising the
amino acid sequences HMYPVR (Sequence I.D. No. 1)
and PGLTPEP (Sequence I.D. No. 2).

13. An antibody to a ligand of P-selectin
comprising a fucosylated sialylated glycoprotein
containing glucosamine and galactosamine in the
approximate ratio of 2:1, N-linked
oligosaccharides, O-linked oligosaccharides or
Ser/Thr-linked oligosaccharides containing the core
disaccharide sequence Galβ1-3GalNAcα-Ser/Thr,
sialyl Lewis x antigen NeuAcα2-3Galβ1-4[Fucα1-
3]GlcNAcβ-R, and poly-N-acetyllactosamine
sequences, wherein the ligand has an apparent
relative molecular weight of 120,000 as assessed by
SDS-PAGE under reducing conditions, an apparent
molecular weight of 250,000 as assessed by SDS-PAGE
under non-reducing conditions.

14. The antibody of claim 13 wherein the
antibody is specifically directed against a
carbohydrate-protein component of the ligand.

15. The antibody of claim 13 wherein the
antibody is immunoreactive against a polypeptide
component of the ligand.

16. A method for modulating an inflammatory or
hemostatic response comprising administering in a
pharmaceutically acceptable carrier an effective
amount of an agent selected from the group
consisting of:

58

a ligand for P-selectin comprising a
fucosylated sialylated glycoprotein containing
glucosamine and galactosamine in the approximate
ratio of 2:1, N-linked oligosaccharides, O-linked
oligosaccharides or Ser/Thr-linked oligosaccharides
containing the core disaccharide sequence Galß1-
3GalNAcα-Ser/Thr, sialyl Lewis x antigen NeuAcα2-
3Galß1-4[Fucα1-3]GlcNAcß-R, and poly-N-
acetyllactosamine sequences, wherein the ligand has
an apparent relative molecular weight of 120,000 as
assessed by SDS-PAGE under reducing conditions, an
apparent molecular weight of 250,000 as assessed by
SDS-PAGE under non-reducing conditions carbohydrate
binding to a selectin,

an antibody directed against the protein-
carbohydrate ligand, and

an antibody to the polypeptide component of
the ligand;

wherein the agent inhibits binding of
leukocytes and other cells to P-selectin.

17.   The method of claim 16 wherein the
carbohydrate is selected from the group consisting
of sialyl Le^x, difucosyl sialyl Le^x, and longer
polyfucosylated polylactosaminoglycans.

18.   The method of claim 16 wherein the agent is
administered in an effective amount to inhibit
metastasis of a tumor.

19.   A method for screening for inhibitors of P-
selectin binding comprising:

(a)   adding to P-selectin a ligand for P-
selectin selected from the group consisting of a
fucosylated sialylated glycoprotein containing
glucosamine and galactosamine in the approximate
ratio of 2:1, N-linked oligosaccharides, O-linked

oligosaccharides or Ser/Thr-linked oligosaccharides
containing the core disaccharide sequence Galß1-
3GalNAcα-Ser/Thr, sialyl Lewis x antigen NeuAcα2-
3Galß1-4[Fucα1-3]GlcNAcß-R, and poly-N-
acetyllactosamine sequences, wherein the ligand has
an apparent relative molecular weight of 120,000 as
assessed by SDS-PAGE under reducing conditions, and
an apparent molecular weight of 250,000 as assessed
by SDS-PAGE under non-reducing conditions, a
fragment of the glycoprotein, a polypeptide
component of the glycoprotein and a carbohydrate
component of the glycoprotein

in combination with a compound to be tested
for inhibiting binding of ligand to P-selectin, and

(b)   determining if binding of P-selectin
to the ligand is inhibited by the compound to be
tested.

20.   A method of isolating a nucleic acid having
a sequence encoding a ligand for P-selectin
comprising:

(a)   screening a human cDNA or genomic
library with a labeled probe, wherein the probe is
selected from the group consisting of:

degenerate synthetic nucleic acid molecules
encoding either HMYPVR or PFLTPEP and

antibodies directed against the ligand for
P-selectin or its carbohydrate components or its
polypeptide components, wherein the ligand
comprises a fucosylated sialylated glycoprotein
containing glucosamine and galactosamine in the
approximate ratio of 2:1, N-linked
oligosaccharides, O-linked oligosaccharides or
Ser/Thr-linked oligosaccharides containing the core
disaccharide sequence Galß1-3GalNAcα-Ser/Thr,
sialyl Lewis x antigen NeuAcα2-3Galß1-4[Fucα1-
3]GlcNAcß-R, and poly-N-acetyllactosamine

sequences, wherein the ligand has an apparent
relative molecular weight of 120,000 as assessed by
SDS-PAGE under reducing conditions, an apparent
molecular weight of 250,000 as assessed by SDS-PAGE
under non-reducing conditions, comprises the amino
acid sequences HMYPVR and PGLTPEP, and wherein the
ligand is isolated from the membranes of human
neutrophils or HL-60 cells by a method comprising
solubilizing cell membranes with nonionic detergent
to form a membrane extract, binding the membrane
extract to a first affinity chromatography column
comprising wheat germ agglutinin, eluting the
ligand from the wheat germ agglutinin affinity
column with acetylglucosamine into ligand-
containing fractions, pooling the ligand-containing
fractions, loading the pooled ligand-containing
fractions from the first affinity chromatography
column onto a second affinity chromatography column
containing immobilized P-selectin, eluting the
ligand from the second affinity column with EDTA,
pooling the ligand-containing fractions from the
second affinity column, loading the pooled ligand-
containing fractions from the second affinity
column onto a MonoQ PC high performance liquid
chromatography column, eluting the ligand from the
HPLC column using a gradient of NaCl, and pooling
the ligand-containing HPLC column fractions;

    (b)   identifying clones containing nucleic
acid sequences encoding the ligand by detecting the
presence of the labeled probe; and

    (c)   isolating the nucleic acid encoding
the ligand from the clone.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   C12N15/10   CO7K15/14   A61K37/02   A61K39/395   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   C12N   CO7K   A61K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELL BIOLOGY vol. 118, no. 2 , July 1992 , NEW YORK, USA pages 445 - 456 K. MOORE ET AL. 'Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells.' see the whole document	1,3-11
X	WO,A,92 01718 (REGENTS OF THE BOARD OF THE UNIVERSITY OF OKLAHOMA) 6 February 1992 cited in the application see claims see examples 5,6,8	1,3-11, 13-19
	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 March 1994	08. 04.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 188, no. 2 , 30 October 1992 , ORLANDO FL, USA pages 760 - 766 C. STEININGER ET AL. 'The glycoprotease of Pasteurella haemolytica A1 eliminates binding of myeloid cells to P-selectin but not to E-selectin.' see the whole document	1,11,19
X	WO,A,92 16612 (NEW ENGLAND MEDICAL CENTER HOSPITALS) 1 October 1992 see claims	16-19
A	WO,A,91 06632 (NEW ENGLAND MEDICAL CENTER HOSPITALS) 16 May 1991 see claims	16-19
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 268, no. 17 , 15 June 1993 , BALTIMORE MD, USA pages 12764 - 12774 K. NORGARD ET AL. 'Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides.' cited in the application see the whole document	1,3-11

2

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 16-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9201718	06-02-92	AU-A-	8620791	18-02-92
		CA-A-	2086323	18-01-92
		EP-A-	0544815	09-06-93
		JP-T-	5509330	22-12-93
		US-A-	5198424	30-03-93
WO-A-9216612	01-10-92	EP-A-	0575557	29-12-93
WO-A-9106632	16-05-91	CA-A-	2067250	21-04-91
		EP-A-	0496832	05-08-92
		JP-T-	5504470	15-07-93

Form PCT/ISA/210 (patent family annex) (July 1992)